Regulation of Sugar Transport and Signaling in Rice

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-Where there's a will, there's a way.

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Abbreviations

ABA	abscisic acid
ABRE	ABA-responsive element
CCCP	carbonyl-cyanide-*m*-chlorophenyl-hydrazone
2,4-D	2,4-dichlorophenoxyacetic acid
GA	gibberelline
GA_3	gibberellic acid
GARE	GA-responsive element
GUS	β-glucronidase
Lea	late embryogenesis abundant
LUC	luciferase
MST	monosaccharide transporter
3OMG	3-*O*-methyl glucose
Rab	responsive to abscisic acid
RAmy	rice α-amylase
SEs	sieve elements
SUT	sucrose transporter

CONTENTS

Chapter 1

General Introduction

Sucrose represents the major transport form of photosynthetically assimilated carbon in plants. Sucrose synthesized in mesophyll cells is exported via the phloem, the long-distance distribution network for assimilates, to supply non-photosynthetic tissues and organs, including the entire below-ground portion of the plant, with energy and carbon resources. To achieve the long-distance transport of sugars from source to sink organs, land plants have evolved the vascular system (the phloem). Indeed, as much as 80 % of the carbon acquired in photosynthesis is transported in the vascular system to non-photosynthetic, heterotrophic organs and the most abundant compound in the phloem sap of most plant species is the sucrose [Zimmermann and Ziegler 1975].

The long-distance transport of sucrose depends on a family of proteins that act as sucrose carriers. Many histochemical observations revealed a specific localization of the sucrose transporter proteins in sieve elements (SEs). Furthermore, the analysis of transgenic plants transformed with antisense constructs has demonstrated that sucrose transporter is essential for phloem loading and allocation of sucrose to sink organs [Riesmeier et al. 1994, Lemoine et al. 1996, Bürkle et al. 1998, Scofield et al. 1999]. These results indicate that phloem loading occurs in SEs by transmembrane uptake of sucrose directly from the apoplastic region by sucrose transporters, therefore which mediates a pivotal step in assimilate partitioning.

Besides sucrose functions as a main phloem loader in SEs, sugars also serve as a signal to regulate expression of specific genes in a variety of different tissues [Chiou and Bush 1998, Umemura et al. 1998, Sheen et al. 1999]. In rice, modulation of α-amylase genes by sugars and other metabolites is well described [Hwang et al. 1998, Umemura et al. 1998, Yu et al. 1996]. At least ten genes encode for α-amylase isoforms in rice [Mitsui et al. 1996], but two of them are strongly under the control of sugar level [Yu et al. 1991, 1996, Karrer and Rodriguez 1992]. While gibberellic acid (GA_3) plays a major role in the up-regulation of α-amylase genes in the aleuron tissues of cereal seeds, sugars may

down-regulate most α-amylase genes in the embryos [Perata et al. 1997]. Some experimental data suggest the existence of hormonal- and sugar-signaling mechanisms that can reciprocally influence their transduction pathways [DeWald et al. 1994, Zhou et al. 1998, Bustos et al. 1998, Perata et al. 1997], but evidence for interaction between those pathways is not sufficient.

In this dissertation I analyzed the regulatory mechanisms of sugar transport and signaling, including the relation to hormonal effects. The embryo of cereal grain represents a useful experimental system for studying the interaction between phytohormones and sugars. I used the rice embryos dissected from the endosperm which are known to be glucose responsive tissues, well characterized in their response to gibberellins and sugars as main material for my doctoral research works. I am convinced that an important aspect of my work is the use of this *in planta* system.

In Chapter 2, I have investigated the sugar-repressive *cis*-acting elements for the promoter activity of the rice α-amylase gene, *RAmy3D*, using a transient expression system of rice embryos. The *RAmy3D* is well known to be strongly induced under sugar starvation conditions but not by GA [Yu et al. 1991, 1996, Karrer and Rodriguez 1992]. The expression of this gene is possibly modulated through a hexokinase-based sugar-sensing mechanism [Umemura et al. 1998]. Using 5' flanking deletion and site-directed mutagenesis techniques for the nucleotide sequence of the *RAmy3D* promoter, I revealed that consensus sequences of G motif (TACGTA) and TATCCA T/C motif (GATA motif as its antisense sequence) are responsible for sugar repression. I also demonstrated by *in situ* hybridization technique that the sugar repression of rice α-amylase gene *RAmy3D* takes place in scuteller epithelium cells of callus-forming rice embryos.

In Chapter 3, I have investigated the cross-talk between hormone- and sugar-signaling. I revealed that exogenously applied glucose to rice and barley embryos negatively affects the endogenous ABA content and represses the promoter activity of the ABA-inducible *Rab16A* gene, but no direct influence to

the expression of rice α-amylase gene *RAmy3D* by this modulation of the ABA content. The reduced *Rab16A* gene expression in barley embryos is partially due to the destabilization of its mRNA by exogenously applied glucose. Both *Rab16A* and *RAmy3D* genes have a G motif sequence possessing the conservative core sequence (ACGT), this indicate that variable border sequences of ACGT might be responsible for distinct response to ABA. It is suggested the putative *trans*-acting factor(s) responsive to glucose signaling may bind to both G motifs of *Rab16A* and *RAmy3D* genes to repress their transcription, but the factor(s) responding to ABA are able to bind to the G motif of the *Rab16A* gene to promote its transcription.

Increasing evidence shows that sugars can act as signals affecting plant metabolism and development. A putative sugar sensor in plant cells may be hexokinase [Jang et al. 1997, Umemura et al. 1998]. In yeast, two unusual glucose transporters appear to function as glucose sensors that generate an intracellular glucose signal [Özcan et al. 1996]. It may be speculated that similar sensor systems exist in multicellular organism like higher plants, however, they could not identify so far. By reason of these morphogenically and physiologically complicated characteristics, great numbers of the transporter which catalyzes sugar transport might be involved in this process.

In Chapter 4, I have isolated three cDNA clones encoding monosaccharide transporters (*OsMST1-3*) from rice EST (Expressed Sequence Tag), and characterization and expression analysis investigated. The result from histochemical observation indicate that *OsMST3* gene show the specific expression in the cell which have thickened cell wall. These results suggest that OsMST3 is involved in accumulation of monosaccharides required for cell wall synthesis at the stage of cell thickness.

Finally, I have totally discussed all investigations described in this dissertation in Chapter 5. Little is known how consecutive dynamic system of sugar transport, sugar sensing and subsequent signal transduction is carrying out in plant cells. I am convinced that my data provide an useful piece of information to

elucidate such a complex signaling network.

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Chapter 2

Promoter elements required for sugar-repression of the *RAmy3D* gene for α-amylase in rice

2. 1 Introduction

During germination of cereal grains, α-amylases (EC 3.2.1.1) play a key role in the mobilization of the energy reserves constituted by insoluble starch granules [Jones and Jacobsen 1991, for review]. The enzyme synthesized during the germination of cereal seeds, catalyzes the hydrolysis of the α-1,4 glucan bonds of the starch molecule. It is commonly accepted that, even thought other amylolytic enzymes participate in the process of starch breakdown, the contribution of α-amylase is the prerequisite for the initiation of this process [Perata et al. 1992, for references].

α-Amylase is not present in the dry cereal seed, but is rapidly induced by the action of gibberellins (GAs), produced by the embryo, triggering α-amylase gene regulation in the aleurone layers. The induction of α-amylase by GA in cereal grains and the counteractive role of ABA on the same process represents a classical model system for studying the mode of action of GA. Beside the aleurone layers, also the scutellum plays an important role in the production of α-amylase [Sugimoto et al. 1998].

In rice, modulation of α-amylase genes by carbohydrates and other metabolites is well described [Yu et al. 1991, 1996, Karrer and Rodriguez 1992, Huang et al. 1993, Umemura et al. 1998]. At least ten genes encode for α-amylase isoforms in rice [Huang et al. 1990, Mitsui et al. 1996], but two of them are strongly under the control of sugar level, namely *RAmy3D* and *RAmy3E* [also identified as *Amy3D* (or αAmy3) and *Amy3E* (or αAmy8)] [Yu et al. 1991, 1996, Karrer and Rodriguez 1992], although also the GA-inducible *RAmy1A* gene is modulated by sugars [Morita et al. 1998]. Indeed, while GA plays a major role in the up-regulation of α-amylase genes in the aleurone tissues in cereal seeds, carbohydrates may down-regulate most α-amylase genes in the embryos [Perata et al. 1997].

I used a transient expression system of rice embryos to study the sugar-repressive *cis*-acting elements for the activity of the *RAmy3D* promoter. My data indicate that consensus nucleotide sequence of G and GATA motifs are important for the sugar repression of the gene.

2. 2 Materials and methods

Preparation of rice embryos

Rice seeds (*Oryza sativa* , cv. Notohikari) were sown in petri dishes containing liquid Murashige-Skoog salt mixture and 2 mg/L 2,4-D. Seeds germinated on this medium show enlarged scutellar side (where α-amylase is expressed *in vivo*, see Figure 2-2), allowing an accurate targeting of the gold particles into the scutellum. All the subsequent procedures were performed as described by Umemura et al. (1998).

Chimeric gene constructs

Using the polymerase chain reaction technology, *Hind*III and *Xho*I restriction endonuclease sites were created at the 5' flanking region (-422, -222, -172 and -122 to -65) of the *RAmy3D* gene from the rice genomic clone (λOSg1A). The nucleotide sequence and other characteristics of the gene have been reported before [Huang et al. 1990]. The amplified promoter was attached using the *Hind*III and *Xho*I restriction endonuclease sites of a truncated minimal (-46) cauliflower mosaic virus (CaMV) 35S promoter to the sequence coding for the *Escherichia coli* β-glucuronidase (*gusA*) gene with a modified ATG initiation codon. The first intron from the castor bean catalase gene was inserted into the 5' untranslated sequence [Tanaka et al. 1990]; this construct (*RAmy3D* promoter / -46 of CaMV 35S promoter / first intron of catalase gene / *gusA* /pUC19) is identified as *RAmy3D-GUS*. As an internal standard, I used the *35S-LUC* clone (pREXΦLUC), a construct of the 35S promoter, Ω sequence and first intron of a gene for phaseolin fused with luciferase gene (*LUC*) [Mitsuhara et al. 1996] gifted from Dr. Hirochika. The *35S-LUC* construct expression in rice embryo was unaffected by the sugars and other chemicals used in my experiments.

Transient expression system

Unless differently stated, all experiments were performed with particle-bombardment co-delivery of *RAmy3D-GUS* and *35S-LUC* for data normalization described by Umemura et al. (1998).

Extraction and assays of samples for GUS and LUC activities were performed as described by Lanahan et al. (1992) but incubations for GUS assays were 1 h long. Typical LUC activities were in the range 200,000-500,000 RLU [relative light units] (background from non-transformed tissue was 50-100 RLU). In order to allow easy comparison of the data presented in the different Figures, data were expressed as "Relative GUS/LUC activity (%)" with respect to the control (relative activity = 100).

Sugar assay

Extraction of plant material, recovery experiments and sugar assays for sucrose, glucose and fructose were performed as enzyme coupling method to monitor the reduction of NAD [Guglielminetti et al. 1995].

In situ hybridization

Callus-forming rice embryos were fixed in FAA (formalin-acetic acid-50% ethanol [1:1:18]) for 24 h at 5 °C. After dehydration in a graded 2-methyl-2-propanol series, samples were embedded in PARAPLAST (Oxford Labware, St. Louis, MO) and sectioned at 10 µm by rotary microtome, and applied on slide glasses treated with 3-aminopropyltrichlorosilane (Shinetsu Chemicals, Tokyo, Japan). Digoxygenin-labeled RNA probe was prepared from the coding region of rice α-amylase gene, *RAmy3D* cDNA clone (pOS137). Probes were degraded to a mean length of 150 bp by incubating in alkali at 60 °C. *In situ* hybridization was performed according to Kouchi and Hata (1993). Hybridization signals were also detected according to Kouchi and Hata (1993). Hybridization signal was not detected when sense probes were used. Accordingly, only results obtained using the antisense probe were shown.

2. 3 Results

RAmy3D *gene transcript is exclusively expressed in sugar-depleted scutellar epithelium cells of rice embryo*

It is well known that α-amylase activity in cereal seeds is detectable in the scutellar epithelium of the embryo and in the aleurone layer of the endosperm [Okamoto and Akazawa 1979]. *In situ* hybridization techniques revealed that mRNA for rice α-amylase gene *RAmy1A*, a GA inducible high pI group gene, is initially detected in the scutellar epithelium and appeared in the aleurone layer at later stage of germination [Sugimoto et al. 1998, Ranjhan et al. 1992]. The rice α-amylase gene *RAmy3D* is known to be induced under sugar starvation condition but not by GA in the isolated embryos and suspension cultured cells of rice [Yu et al. 1991, 1996, Karrer and Rodriguez 1992, Umemura et al. 1998, Yamaguchi et al. 1996].

Rice embryos excised from the endosperm of a germinating seedlings contain their own carbohydrates. Among them, glucose (arising from starch degradation in the endosperm), sucrose (synthesized in the scutellum), and fructose (derived from sucrose degradation) can down-regulate the *RAmy3D* gene expression. I measured the levels of glucose, fructose and sucrose in the callus-forming embryos after excision from the endosperm (condition 1 in Figure. 1) as well as after 1- and 3-d incubation of the excised embryos on sugar-free medium (i.e., sugar starvation treatment, conditions 2 and 3) and on medium containing 90 mM glucose for 2 d after 1-d sugar starvation (condition 4). The results showed that rice embryos contain sucrose, glucose and a relatively lower level of fructose, and that incubation on the sugar-free medium rapidly leads to a decrease in the endogenous content of sucrose and, mainly, glucose.

I performed *in situ* hybridization of callus-forming rice embryos with antisense probe to demonstrate the location and timing of the sugar repression of *RAmy3D* gene (Figure 2-2). The hybridization signal for the *RAmy3D* mRNA

can not be detected in the embryos immediately after excision from the endosperm (Figure 2-2A), where the cells show a high level of endogenous carbohydrate contents (see condition 1 in Figure 2-1). Instead I was able to detect the signal in the embryos after 1- or 3-d sugar starvation treatment (Figure 2-2B and C). The activation of the mRNA transcription was reversibly suppressed by 2-d glucose treatment after 1-d starvation (Figure 2-2D). Magnified figures revealed that the signal for *RAmy3D* transcript is detectable in the outer-surface cell layers of the embryo (Figure 2-2E) which are originally derived from the scutellar epithelium. The signal is completely repressed in newly divided cells under sugar-rich condition (Figure 2-2F). Interestingly, the signal was also detected in the vascular cells under sugar starvation (Figure 2-2C). Starch granules visualized by PAS staining completely disappeared in the cells under sugar starvation (Figure 2-2G), whereas developed again after glucose treatment (Figure 2-2H), indicating a good correlation between sugar starvation and disappearance of starch granules.

Sugar-repressive cis*-acting elements of* RAmy3D *promoter are involved in consensus sequences of G motif and TATCCA T/C motif*

Addition of carbohydrates to the incubation medium of transformed rice embryos resulted in repression of the *RAmy3D* promoter activity (Figure 2-3, -422/-65 construct). Embryos were dissected from seedlings, transformed with 5' deleted promoter of *RAmy3D-GUS* co-delivered with *35S-LUC* by particle bombardment, and transferred to sugar-free medium (control), or to medium containing 90 mM sucrose for 2 d. As shown in Figure 2-3A, the *RAmy3D* promoter activity visualized by GUS staining is repressed by sugar. While deletion of the -422 to -172 sequence had no effect on the promoter activity, deletion of the -422 to -122 fragment resulted in a dramatically reduced promoter activity under sugar starvation condition (control of -122/-65 in Figure 2-3B). These results from the 5' deletion analyses suggest that the 50-bp nucleotide

sequence from -172 to -123 of the *RAmy3D* promoter has a responsibility for the sugar repression.

Experiments were performed using *RAmy3D* promoters after mutagenesis of the 50-bp nucleotide sequence from -172 to -123 (Figure 2-4). Mutagenesis of 8-bp sequences at -161 to -154 (M2), -151 to -144 (M3) and -131 to -124 (M5) results in a significant reduction of the promoter activity under sugar starvation, whereas no effect at -171 to -164 (M1) and -141 to -134 (M4). These results suggest that the sequences at -161 to -144 (M2 and M3) and -131 to -124 (M5) are involved in the sugar-responsive *cis*-acting elements. From comparison between those sequences and registered *cis*-acting motif sequences on the data base, I found consensus sequences designated as G motif (consensus CACGTG, -154TACGTG-149 for *RAmy3D*) and TATCCA T/C motif (-131TATCCAT-125) (Figure 2-5). Both nucleotide sequences might be therefore important for the expression of the *RAmy3D* gene under sugar starvation.

2. 4 Discussion

Sugar repression of α-amylase gene expression

Rice α-amylase genes, *RAmy3D* and *3E* (also *Amy3D* and *Amy3E*) are mainly under sugar control, with phytohormones playing little if any role [Karrer and Rodriguez 1992]. I demonstrated by *in situ* hybridization that the sugar repression of rice α-amylase gene *RAmy3D* takes place in scutellar epithelium cells of callus-forming rice embryos (Figure 2-2). Although the sugar repression has been thought to be restricted to the *3D* and *3E* genes [Karrer and Rodriguez 1992, Sheu et al. 1996], the *RAmy1A* gene, which is clearly under hormonal control in the aleurone [Itoh et al. 1995], is also affected by sugar regulation but to a smaller extent when compared with that of *RAmy3D* [Morita et al. 1998]. Furthermore, Perata et al. (1997) have reported that even barley α-amylase genes (both high and low pI group) are under sugar control in the embryo (but not in the aleurone), indicating that α-amylase, a key enzyme of starch degradation in cereal seedlings, may be generally under sugar control in the embryos.

Sugar-repressive cis*-acting elements in α-amylase and other plant genes*

Preliminary promoter characterization of the *RAmy3D* gene using transgenic cell cultures of rice have been reported by Huang et al. (1993). DNase I footprinting analyses using binding activity of a nuclear protein from the suspension-cultured cells of rice to the *RAmy3D* promoter sequence revealed three protein-binding regions, each of these protein-binding sequences contained the GCCCG G/C CG motif [Mitsunaga et al. 1994]. These heptameric binding motifs are located at -269, -243 and -209 of the gene. From the present demonstrations, however, those binding motifs seem to be not critical for the sugar-repression of the *RAmy3D* gene. Site-directed mutagenesis of 8-bp sequences at -161 to -154 (M2), -151 to -144 (M3) and -131 to -124 (M5) results in a significant reduction of the promoter activity under sugar starvation (Figure

2-4), indicating that those nucleotide sequences are associated with sugar repression. Finally I found consensus sequences designated as G motif (consensus CACGTG, -154TACGTG-149 for *RAmy3D*) and TATCCA T/C motif (-131TATCCAT-125) in the regions. Both motif sequences are probably important for the expression of the *RAmy3D* gene under sugar starvation. G motif, however, could not be found in the promoter sequence of *RAmy1A* around the position, although the gene is also under sugar control, whereas TATCCA T/C motif is able to be found in both the promoter sequences at the same position (see Figure 2-5). TATCCA T/C motif contains GATA motif as its antisense sequence, especially TATCcaTATC sequence for *RAmy3D* means GATAtgGATA sequence as its antisense sequence, which has been reported in petunia *cab22L* gene promoter as a light-regulated motif [Gilmartin et al. 1990]. Instead of G motif for the *RAmy3D*, GARE (TAACAAA) is able to be found in the *RAmy1A*, which is well characterized as a GA-responsive *cis*-acting element [Lanahan et al. 1992, Skriver et al. 1991, Gubler and Jacobsen 1992, Gubler et al. 1995] (Figure 2-5).

Hwang et al (1998) recently reported the *cis*-elements required for rice α-amylase *Amy3D* (identified as *RAmy3D* in this experiment) expression during sugar starvation [Hwang et al. 1998]. Their functional promoter analyses using electroporated rice protoplasts revealed that three sequence having the greatest effects on *Amy3D* gene expression included the CGACG element and additional two *cis*-elements that are reported in this experiment, i.e., the amylase element (TATCCA T/C motif) and G box-related element. These compatible results strongly suggest the specific sugar-repressive *cis*-elements of the *RAmy3D* promoter. For the first CGACG element, however, the site-directed mutagenesis (M1) in this experiment (tagCG from CGACG, see Figure 2-4) showed no reduction of the promoter activity under sugar starvation, indicating that distinct experimental systems (protoplasts vs. embryos) may lead diverse results.

Three conserved sequences for the promoter region of most GA-inducible α-amylase genes in cereals have been reported, i.e., pyrimidine motif (CCTTTT)

at diverse position, GARE motif at around -150 position and TATCCA T/C motif at around -120 position (for example, *RAmy1A* promoter in Figure 2-5, [Huang et al. 1993, Gubler and Jacobsen 1992]). Functional promoter analyses for *RAmy1A* gene revealed that both pyrimidine and GARE motifs are partially involved in the sugar repression, but not TATCCA T/C motif [Morita et al. 1998]. Overall results for sugar-repressive *cis*-acting elements compared with *RAmy3D* and *RAmy1A* promoter might indicate that sugar repression of rice α-amylase genes is not due to conservative *cis*-acting motifs among the promoters but common signaling process(es) for sugar sensing in the embryos. Indeed Perata et al. [Perata et al. 1997] have recently reported that sugar and hormonal signaling interact in the regulation of gibberellin-induced α-amylase gene expression in barley embryos.

Little is known about the sugar-repression mechanism(s) underlying gene regulation by carbohydrate in plant systems [Koch 1996, Graham 1996, for review]. Functional promoter analyses using promoters for the gene of glyoxylate cycle enzyme in cucumber have reported nucleotide sequence IMH2 (AA A/C CCCA C/A CCT) as a putative sugar-response *cis*-acting element [Sarah et al. 1996, Reynolds and Smith 1995, De Bellis et al. 1997].

G motif and GATA motif as a sugar-response cis*-acting element*

G motif is a hexameric motif, CACGTG, found in many diverse plant genes. This sequence functions as a *cis*-acting promoter element, and first characterized on the 5' flanking region of the light-regulated ribulose 1,5-bisphosphate small subunit (*RBCS*) genes. After this report, many groups have shown that the G motif sequence resides in the promoters of many genes that are switched on in response to quite diverse stimulatory pathway, i.e., light, anaerobiosis, *p*-coumaric acid and phytohormones such as abscisic acid, ethylene and methyl jasmonate [Menkens et al. 1995, for review]. My results from functional promoter analyses of rice α-amylase gene, *RAmy3D*, suggest that G

motif-like element also responds to endogenous carbohydrates levels (Figure 2-4). In each of the promoters reported previously, the G motif resides in a unique DNA context and additional elements are critical to the appropriate response. In the case of *RAmy3D* gene, additional GATA motif sequence may be critical to the specific sugar response. GATA (or -I) motif has been also identified as a light-regulated *cis*-acting element [Gilmartin et al. 1990, for review]. Indeed, detailed experiment by Puente et al. (1996) indicate that combination of G and GATA motifs can serve as minimal autonomous promoter determinants which integrate light and developmental signals and modulate promoter activity. Cellular level of carbohydrates, end product of photosynthetic function, is probably trigger signal(s) involved in the light-regulation. Indeed the expression of most photosynthesis genes is regulated by metabolizable carbohydrates [Jang et al. 1997]. Further study will be needed to test this speculation.

2. 5 Summary

There is increasing evidence showing that cereal α-amylase gene expression is controlled not only by the classical hormonal regulation, but also by feed-back sugar repression. I demonstrated by *in situ* hybridization that the sugar repression of rice α-amylase gene *RAmy3D* takes place in scutellar epithelium cells of callus-forming rice embryos. I also used a transient expression system to study the *cis*-acting elements involved in the sugar repression of the *RAmy3D* promoter activity. Site-directed mutagenesis of the 50-bp nucleotide sequence from -172 to -123 revealed that consensus sequences of G motif (TACGTA) and TATCCA T/C motif (GATA motif as its antisense sequence) are responsible for sugar repression. The promoter sequences required for sugar repression are reported and discussed.

2. 6 References

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Figures

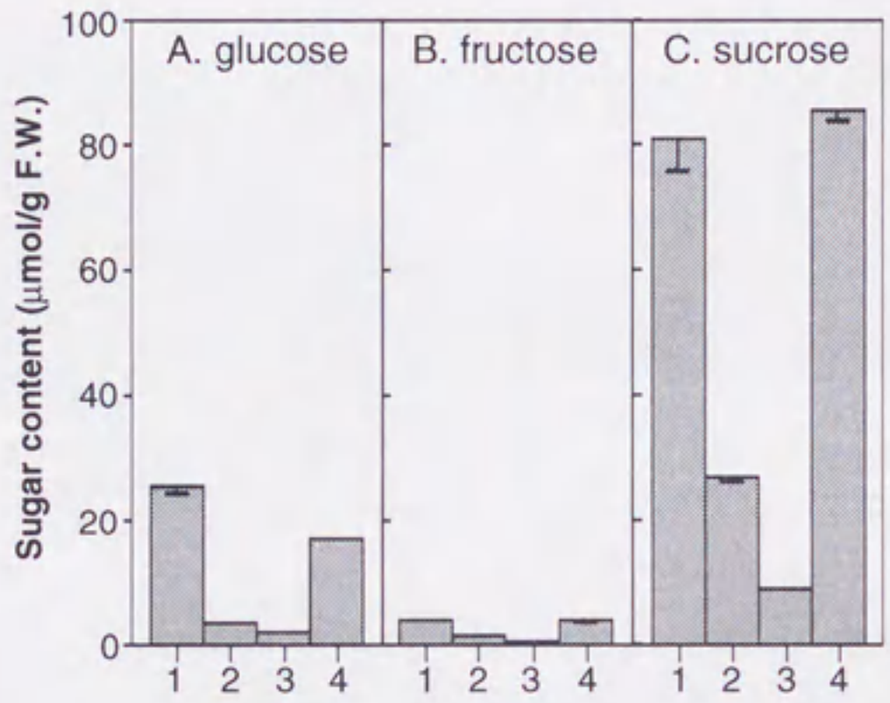

Figure 2-1 Endogenous carbohydrate content in callus-forming rice embryos.

A, glucose; **B**, fructose; and **C**, sucrose. Five embryos excised from the endosperm (condition 1) were incubated on sugar-free medium for 1 d (condition 2). After that starvation, embryos were incubated on sugar-free medium for additional 2 d (condition 3) or on medium containing 90 mM glucose for 2 d (condition 4). Data are mean ± SE (n=3).

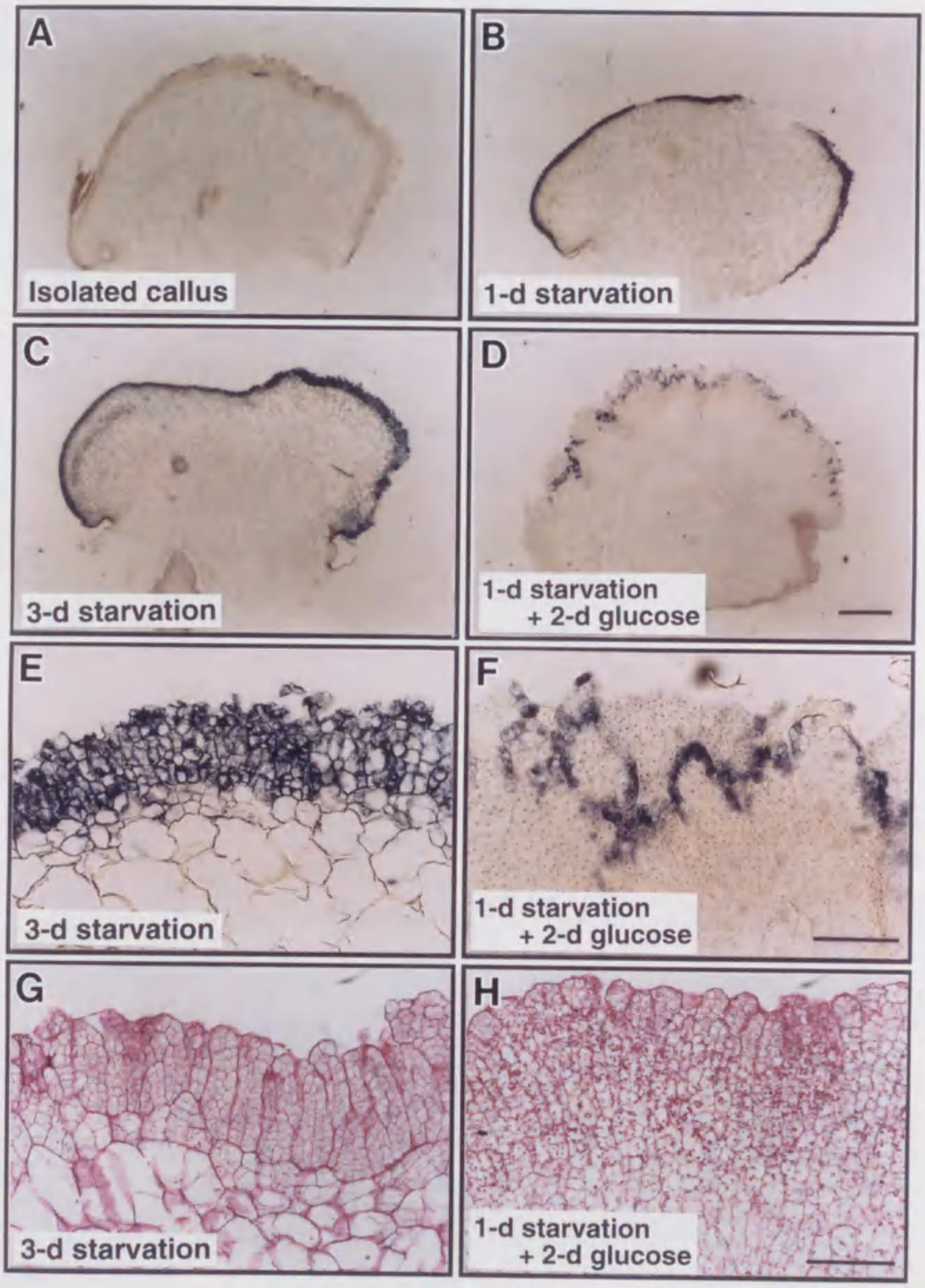
A
Isolated callus
B
1-d starvation
C
3-d starvation
D
1-d starvation
+ 2-d glucose
E
3-d starvation
F
1-d starvation
+ 2-d glucose
G
3-d starvation
H
1-d starvation
+ 2-d glucose

Figure 2-2 Histochemical observations in callus-forming rice embryo.

A-F: Localization of *RAmy3D* mRNAs in rice embryo by *in situ* hybridization. Embryos excised from the endosperm (**A**, also see condition 1 in Figure 2-1) were incubated on sugar-free medium for 1 d (**B**, condition 2). After the starvation treatment, embryos were incubated on sugar-free medium for additional 2 d (**C**, condition 3) or on medium containing 90 mM glucose for 2 d (**D**, condition 4). **E** and **F** are magnified figures of the outer-surface cells of panels **C** and **D**, respectively. Reduction of endogenous carbohydrate levels promotes the expression of *RAmy3D* gene exclusively in cells of the scutellar epithelium.

G and **H**: Starch granules visualized by Periodic acid-Schiff (PAS) staining in the same embryos as panels **E** and **F**. Starch granules are stained as dark pink particles. Scale bars in A to **D**= 0.5 mm, **E** to **H**= 0.1 mm.

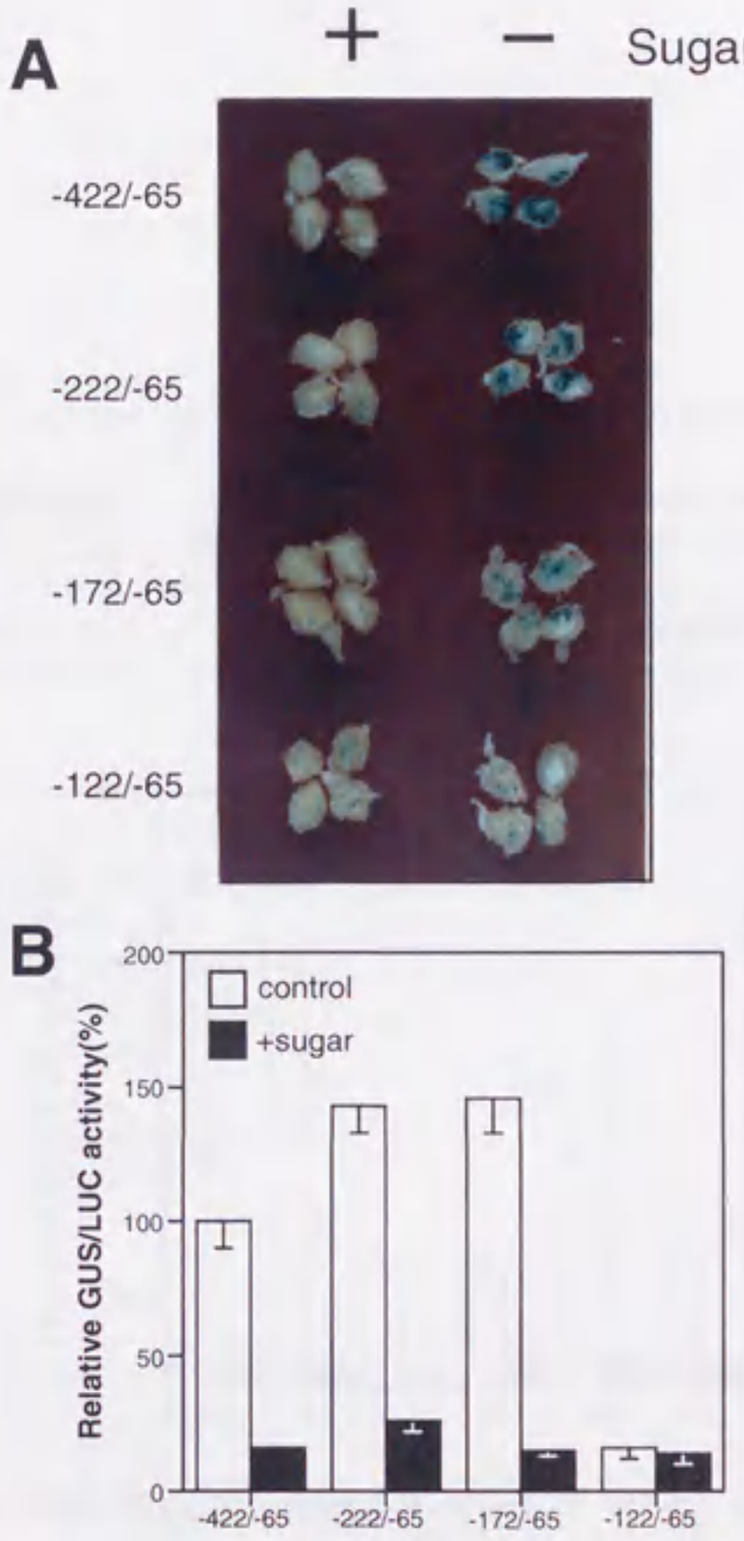

Figure 2-3 Effect of 5' flanking deletion on sugar repression of *RAmy3D* promoter activity.

Rice embryos treated for 1 d on sugar-free medium were transformed by bombardment with 5' deleted promoter (-422, -222, -172 and -122) of *RAmy3D* (/-65)-*GUS* co-delivered with *35S-LUC*. After transformation the embryos were subsequently incubated for 2 d on sugar-free medium (control), or on medium containing 90 mM sucrose (+sugar).

Panel **A**: Visualized promoter activity by GUS staining. Panel **B**: Quantitative data for the promoter activity. Data were normalized by using the *35S-LUC* constructs as an internal standard. Relative GUS/LUC activity is expressed as: -422/-65 construct (control)= 100. Data are mean ± SE (n=3).

```
          -172                                                    -123
RAmy3D    CGCGGCCGAC GCGGCGCCTA CGTGGCCATG CTTTATTGCC TTATCCATAT

M1        CATAATTAGC GCGGCGCCTA CGTGGCCATG CTTTATTGCC TTATCCATAT
M2        CGCGGCCGAC GTAATATTCA CGTGGCCATG CTTTATTGCC TTATCCATAT
M3        CGCGGCCGAC GCGGCGCCTA CACAATTGCG CTTTATTGCC TTATCCATAT
M4        CGCGGCCGAC GCGGCGCCTA CGTGGCCATG CCCCGCCATC TTATCCATAT
M5        CGCGGCCGAC GCGGCGCCTA CGTGGCCATG CTTTATTGCC TCGCTTGCGT
```

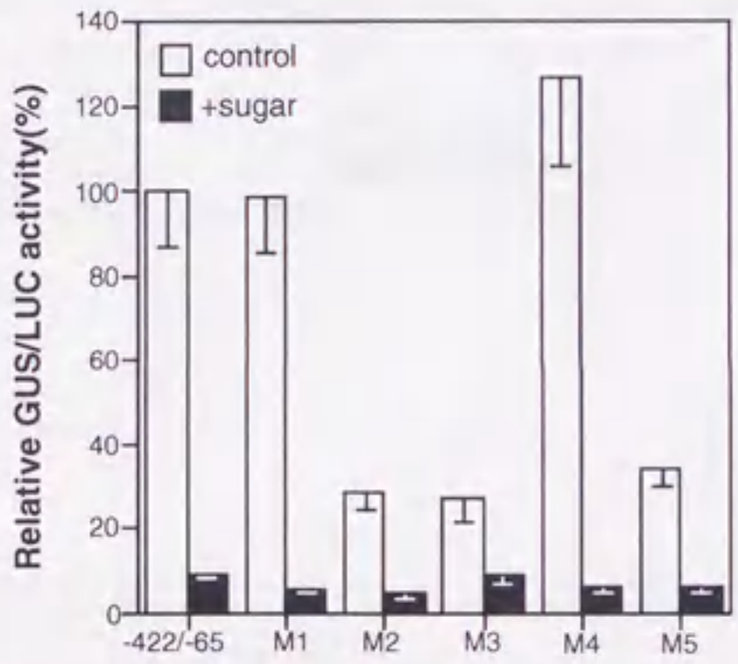

Figure 2-4 Effect of site-directed mutagenesis of 50-bp nucleotide sequence (-172 to -123) of *RAmy3D* promoter on sugar repression of the promoter activity.

Diagram showing native and mutated sequences at the position of -171 to -164 (M1), -161 to -154 (M2), -151 to -144 (M3), -141 to -134 (M4) and -131 to -124 (M5) sequences are shown in the upper panel. Rice embryos treated for 1 d on sugar-free medium were transformed by bombardment with site-directed mutagenesis promoter of *RAmy3D* (-422/-65)-*GUS* co-delivered with *35S-LUC*. After transformation the embryos were subsequently incubated for 2 d on sugar-free medium (control), or on medium containing 90 mM glucose (+sugar). Data were normalized by using the *35S-LUC* construct as an internal standard. Relative GUS/LUC activity is expressed as: -422/-65 native construct (control)= 100. Data are mean ± SE (n=3).

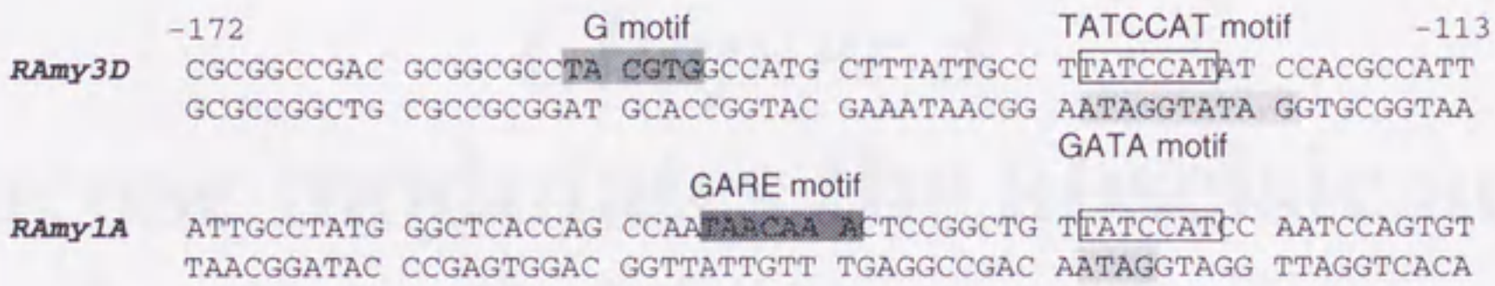

Figure 2-5 Comparison of regulatory *cis*-acting elements between *RAmy3D* and *RAmy1A* promoter sequence (-172 to -113).

Putative *cis*-acting elements between the two promoter sequences indicate as: TA CGTG, G motif for *RAmy3D*; TATCCAT, TATCCA T/C motif for both promoters, contains GATA motif sequence as antisense direction especially GATAxxGATA sequence for *RAmy3D*; TAACAAA, GARE (gibberellin response element) for *RAmy1A*. For specific characters for these motifs, see in the text.

Chapter 3

Glucose modulates the abscisic acid-inducible *Rab16A* gene in cereal embryos

3. 1 Introduction

Sugars are an important source of energy and carbon skeletons for plant growth and development but also act as signaling molecules influencing developmental and metabolic processes [Koch 1996]. The sugar-sensing mechanisms modulating gene expression have received considerable attention in the recent years [Jang et al. 1997, Smeekens 1998].

Increasing evidence indicates the existence of cross-talk between hormone- and sugar-signaling. Auxin counteracts the activation of the vegetative storage protein gene triggered by sugars in soybean [DeWald 1994], sugars and cytokinin interact during tobacco leaf senescence [Wingler et al. 1998], and cross-talk between glucose and ethylene signaling has been demonstrated [Zhou et al. 1998]. Sugars repress the GA-signaling leading to the induction of α-amylase in barley (*Hordeum vulgaris* L. var Himalaya) embryos [Perata et al. 1997]. Sugar induction of sporamin and β-amylase can be mimicked by abscisic acid (ABA) [Ohto et al. 1992], and the expression of the β-phaseolin promoter is influenced by both ABA and sucrose [Bustos et al. 1998]. These data suggest the existence of hormone and sugar signaling mechanisms that can reciprocally influence their transduction pathways.

The embryo of cereal grains represents a useful experimental system for studying the interaction between hormones and sugars. Perata et al. (1997) investigated the effects of sugars on the gibberellic acid (GA_3)-induced α-amylase gene expression in barley grains. The results indicate that α-amylase synthesis in the scutellar epithelium of barley embryos is modulated by both sugars and hormones [Perata et al. 1997]. The GA-induced rice α-amylase *RAmy1A* gene was also shown to be repressed in response to exogenously applied glucose, and the GA-response *cis*-acting elements of GARE (TAACAAA) and pyrimidine box (CCTTTT) were demonstrated to be partially involved in the sugar repression [Morita et al. 1998]. Rice α-amylase gene *RAmy3D* is also well known to be

under glucose control [Toyofuku et al. 1998]. Although in cereals, including rice, many α-amylase genes are hormonally modulated with gibberellins playing an inductive role and abscisic acid counteracting the gibberellin effect, the *RAmy3D* promoter does not contain the typical GA-responsive element (GARE), and consequently is not induced by gibberellin [Yamaguchi 1998]. It is unknown if other hormones affect its expression. A question to be addressed is which factor is responsible for the sugar-repression of *RAmy3D* promoter activity and if ABA is somehow involved in the glucose-triggered repression of the *RAmy3D* gene. Indeed, the *RAmy3D* promoter region has an ACGT sequence, responsible for its glucose repression, which is also found in the promoter region of ABA-modulated genes as part of the ABA-responsive elements (ABREs).

ABA-modulated genes are also expressed in cereal embryos. ABA is crucial for the adaptation to environmental stresses such as drought, cold, and high salinity, but is also involved in the physiology of grain development and germination [Bray 1993, Ingram and Bartels 1996, Jensen et al. 1998, Neill et al. 1987, Zeevaart and Creelman 1988]. In most cases, these processes are associated with the expression of genes modulated by ABA. During the late stages of seed development, the well-characterized ABA-induced *Lea* (late embryogenesis abundant) mRNAs and proteins accumulate [Baker et al. 1988, Skriver and Mundy 1990]. From sequence and functional analysis of the promoters of *Lea* and *Lea*-related genes, ABREs with core sequence ACGT have been identified [Busk and Pagès 1998, Guiltinan et al. 1990, Izawa et al. 1993, Mundy and Chua 1988, Oeda et al. 1991, Williams et al. 1992]. One of the *Lea*-related genes, the rice *Rab16A* gene, shows strong expression in the embryos during the late stage of grain development as well as in response to ABA and osmotic stress in vegetative tissues [Ono et al. 1996, Yamaguchi-Shinozaki et al. 1989].

Perata et al. (1997) showed that a treatment with glucose reduced the ABA content of barley embryos. It is not known whether glucose treatment affects the

expression of the ABA-modulated *Rab16A* gene, and if its effects on ABA content could be responsible for an altered expression of the *Rab16A* gene. I describe in this Chapter the effects of glucose on the expression of the *Rab16A* gene in the embryo of cereal grain. Both rice and barley embryos were used as experimental systems; both are known to be glucose responsive tissues, well characterized in their response to gibberellins and sugars [Perata et al. 1997, Toyofuku et al. 1998]. Moreover, rice embryos allowed us to perform transient expression experiments using a homologous expression system. The experimental results revealed that the expression of the *Rab16A* gene is repressed by glucose both at the transcriptional and post-transcriptional level independently from the effects of glucose on ABA content.

3. 2 Materials and methods

Rice callus experiments

Rice grains (*Oryza sativa* L., cv. Notohikari) were sown in petri dishes containing liquid Murashige-Skoog salt mixture [Murashige and Skoog 1962] and 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid). The grains germinated on the medium showed enlarged scutellar and callus development from the epithelium cells after 8 d. The embryos were dissected from the endosperm by carefully removing any starch, which, if degraded, might alter the carbohydrate contents of the embryo during the experiments. I termed this sample as a "callus-forming" embryo of rice. Callus-forming from the single-layer of cells of the scutellar epithelium, the site of α-amylase gene expression *in vivo* [Toyofuku et al. 1998], allowed an accurate targeting of the gold particles. All the subsequent procedures were performed as described by Umemura et al. (1998).

Barley embryo experiments

Barley (*Hordeum vulgare* L.) grains of cultivar Himalaya (1995 harvest, Washington State University, Pullman, WA) were used. Embryos were dissected from sterilized grains (shaking in 5% sodium hypochlorite for 1 h; washing in sterile water with shaking for 2 h) using a scalpel. Only perfectly intact embryos were used, with no starch adhering to the scutellar tissue. Incubation of embryos was carried out in 24-well plastic plates, with each well containing four embryos and 500 µl of 5 mM $CaCl_2$ containing 5 µg of chloramphenicol. The embryos were incubated at 25 °C with vigorous shaking. When used, abscisic acid (ABA) at the concentrations indicated in the figures was added.

RNA extraction and gel blots

RNA extraction was performed by using the aurintricarboxylic acid method, as described by Skadsen (1993), with minor modifications. Northern blots were

performed as described by Perata et al. (1997). Total RNA (20 µg) of each sample was electrophoresed on formaldehyde gel and blotted onto a nylon membrane (Hybond N^+; Amersham). Membranes were prehybridized for 1 h at 65 °C in 0.5 M NaH_2PO_4, 7 % SDS, 1 % PVP (polyvinyl pyrrolidone) and 50 µl/ml salmon sperm DNA. Radiolabeled probes were prepared from gel-purified cDNA inserts by random primer labeling with α-^{32}P-dCTP.
Hybridization was performed at 65 °C in the prehybridization buffer. The membranes were washed one time in 2xSSC (1xSSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 1 % SDS for 15 min and two times in 0.2xSSC and 1 % SDS at 15 min-intervals at 65 °C. Membranes were exposed to X-ray film for 2 d.

Chimeric gene constructs

Using the polymerase chain reaction technology, *Hind*III and *Xho*I restriction endonuclease sites were created at the 5' flanking region (-422) of the *RAmy3D* gene from the rice genomic clone (λOSg1A). The nucleotide sequence and other characteristics of the gene have been reported previously [Huang et al. 1990, Mitsunaga et al. 1994]. The amplified promoter was attached using *Hind*III and *Xho*I restriction endonuclease sites of a truncated minimal (-46) cauliflower mosaic virus (*CaMV*) 35S promoter to the sequence coding for the *Escherichia coli* β-glucuronidase (*gusA*) gene with a modified ATG initiation codon. The first intron from the castor bean catalase gene was inserted into 5' untranslated sequence [Tanaka et al. 1990]; this construct (*RAmy3D* promoter/-46 of CaMV 35S promoter/first intron of catalase gene/gusA/pUC19) is identified as *RAmy3D-GUS* [Toyofuku et al. 1998, Umemura et al. 1998].

Rab16A-GUS gene construct was a gift from Dr. Hattori (Center for Molecular Biology and Genetics, Mie Univ., Japan).

As an internal standard, I used the *35S-LUC* clone (pREXΦLUC), a construct of the 35S promoter fused with luciferase gene (*LUC*) [Mitsuhara et al.

1996], a gift from Dr. Hirochika (National institute of Agrobiological Resources, Tsukuba, Japan). The expression of the *35S-LUC* construct in rice embryos was unaffected by sugars and other chemicals used in my experiments.

Transient expression system

Unless differently stated, all experiments were performed with particle-bombardment co-delivery of *RAmy3D-GUS* or *Rab16A-GUS* and *35S-LUC* for data normalization as described by Umemura et al. (1998).

Bombardment was performed according to the instruction provided by the manufacturer (BioRad) by using a 1100 psi He pressure and the sample holder closer to the gun (5 cm from the stopping screen). The bombardment was repeated twice on each plate containing 30-40 rice embryos. After bombardment, rice embryos were transferred in petri dishes containing Murashige-Skoog salt mixture and 2 mg/l 2,4-D supplemented with/without ABA, fluridone and 90 mM glucose.

Each experiment was repeated 2 to 3 times, on different days, and with freshly prepared new batches of reagents and rice embryos. Each independent experiment consisted of three replicates of 5 embryos each. All repeated experiment gave consistent results. The reported data are means of the obtained results from a representative experiment.

Extraction and assays of samples for GUS and LUC activities were performed as described by Lanahan et al. (1992) but incubations for GUS assays were 1 h long. Typical LUC activities were in the range 200,000-500,000 RLU [relative light units] (background from non-transformed tissue was 50-100 RLU). In order to allow easy comparison of the data presented in the different figures, data were expressed as "Relative GUS/LUC activity %" with respect to the control (relative activity = 100).

Assay of ABA

ABA content in rice and barley embryos was determined by radioimmunoassay by using a highly specific monoclonal antibody as previously described [Vernieri et al. 1989, Walker-Simmons 1990]. The absence of cross-reacting material other then ABA in the extracts was verified by HPLC fractionation of the crude extracts [Perata et al. 1997].

Assay of sugar

Extraction of plant material, recovery experiments and glucose assays were performed as previously described [Guglielminetti et al. 1995].

3. 3 Results

Glucose effects on the endogenous ABA level in rice embryos

I measured the content of endogenous ABA in the callus-forming rice embryos after excision from the endosperm (Treatment 1 in Figure 3-1), as well as after 1- and 3-d long incubation of the excised embryos on glucose-free medium (i.e. endogenous glucose depletion; Treatments 2 and 3 in Figure 3-1) and on a medium containing 90 mM glucose for 2 d after 1 d of glucose depletion (Treatment 4 in Figure 3-1). The ABA content in the embryos after excision from the endosperm (control, Treatment 1) was estimated to be less than 10 ng/g FW. After glucose depletion (Treatment 2 and 3), the ABA content remarkably increased up to 100 ng/g FW. Exogenous glucose treatment (Treatment 4) restored the glucose and ABA content found in the control (Treatment 1).

Glucose affects transcription of an α-amylase gene in rice embryos

Glucose depletion in the rice embryos resulted in an enhanced endogenous ABA content (Figure 3-1). To analyze the effect of ABA on glucose repression of the *RAmy3D* gene, rice embryos were treated with exogenously applied ABA and fluridone, an inhibitor of ABA biosynthesis. A reduction of ABA content after application of fluridone (Treatments 3, 5, and 6 in Figure 3-2) was observed compared with that of embryos incubated without glucose (Treatment 1) or with mannitol application (Treatment 2). A very low ABA content was detected after glucose feeding (Treatments 4 and 6). Northern analysis confirmed that the mRNA level of *RAmy3D* gene is dramatically reduced by glucose application (compare lanes 1 and 2 in Figure 3-3A). Addition of ABA (lane 3) and fluridone (lane 5) to the embryos did not affect the glucose repression of *RAmy3D* gene (compare lanes 3 and 4 and lanes 5 and 6, Figure 3-3A). Performing co-delivery transformation experiment using *RAmy3D-GUS* and *35S-LUC* constructs with particle-bombardment, I analyzed the effect of ABA, glucose, and fluridone on the

promoter activity of *RAmy3D* gene (Figure 3-3B). Glucose repression of the promoter activity was unaffected by the presence of ABA and fluridone (see Treatments 3 and 5 compared with Treatment 1). These results indicate that the starvation-dependent increase in endogenous ABA level does not affect the starvation-dependent promotion of *RAmy3D* gene transcription.

Glucose affects transcription of the Rab16A gene in rice embryos

I examined the effects of glucose on the expression of the *Rab16A* gene in rice embryos. In the absence of exogenous ABA, the high level of *Rab16A* mRNA (lane 2, Figure 3-4A), mirrors the high ABA content of embryos incubated in the absence of glucose (Treatment 1, Figure 3-2). Addition of glucose results in a lower level of both ABA content and *Rab16A* mRNA (Treatment 4, Figure 3-2; lane 1, Figure 3-4A). However, addition of exogenous ABA did not affect the sugar-repression of *Rab16A* (Lanes 3 and 4, Figure 3-4A), suggesting that glucose negatively influences ABA signaling besides ABA synthesis. This was confirmed by treating the rice embryos with fluridone. As shown in Figure 3-2 fluridone reduces the endogenous ABA content to levels comparable to those observed in glucose-treated embryos, but glucose treatment strongly reduced the *Rab16A* mRNA level in the presence of fluridone (Lanes 5 and 6, Figure 3-4A).

I used callus-forming rice embryos as a transient expression system to analyze the promoter activity of *Rab16A* gene. I performed co-delivery transformation experiments of *Rab16A-GUS* and *35S-LUC* genes with particle-bombardment. The presence of glucose negatively affected the promoter activity of *Rab16A* (Figure 3-4B), e.g. the promoter activity of *Rab16A* was reduced by glucose treatment regardless of exogenous ABA application. These results indicate that glucose feeding represses the *Rab16A* gene at the transcriptional level.

Glucose represses Rab16A gene expression in barley embryos

ABA treatment (1 to 10 μM) of barley embryos resulted in a dramatic

increase in the *Rab16A* mRNA level (Figure 3-5A). On the contrary, no expression was observed in the embryos not treated or treated with low (0.1 μM) ABA concentration (Figure 3-5A). Addition of 50 mM glucose to the incubation medium negatively affects the ABA-triggered increase in *Rab16A* mRNA level, with a 60 to 80 % repression detected when glucose was added to the 10 and 1 μM ABA medium, respectively (Figure 3-5B).

I investigated the effects of the glucose treatment on the ABA content in the ABA-treated barley embryos. Mannitol was also used at the same concentration as glucose to exclude a possible osmotic effect. No differences were observed when comparing the ABA level of the ABA-treated embryos with that of ABA+glucose or ABA+mannitol-treated embryos (Figure 3-6B). The results reported in Figure 3-6 can be summarized as follows: i) application of ABA to the embryos resulted in a drastic increase in the *Rab16A* mRNA level, partly reduced when glucose was also present (Figure 3-6A). ii) Neither the glucose nor the mannitol treatments resulted in statistically significant variations in the ABA content of the embryos (Figure 3-6B). Although glucose treatment of barley embryos (not treated with exogenous ABA) results in a lower endogenous ABA content [Perata et al. 1997], the results reported in Figure 6 suggest that the glucose effects on the embryos treated with exogenous ABA are not due to modulation of the endogenous ABA content.

Glucose affects Rab16A *mRNA stability in barley embryos*

I evaluated if glucose treatment results in a destabilization of the *Rab16A* mRNA. Barley embryos, previously incubated for 14 h in an ABA solution, were treated with 50 mM glucose (Figure 3-7). The 0-14 h treatment with ABA resulted in the induction of the *Rab16A* mRNA (the transcript was absent in control embryos not treated with ABA, data not shown). Further treatment with ABA up to 22 h resulted in a slightly higher mRNA level, but transfer to a medium containing ABA and glucose resulted in a decreased *Rab16A* mRNA level, which

was lower than that expected from a repression of the *Rab16A* transcription. A 14-22 h treatment with the transcription inhibitor actinomycin-D did not affect the mRNA level, indicating that the *Rab16A* is stable over the 14-22 h period without glucose treatment (Figure 3-7). These results indicate that glucose reduces the stability of *Rab16A* mRNA in the barley embryos.

3. 4 Discussion

Glucose repression of Rab16A *gene expression*

I demonstrated that the expression of *Rab16A* gene in rice and barley embryos is down-regulated by exogenously applied glucose through repression of transcription (Figure 3-4B) and destabilization of its mRNA (Figure 3-7). The repression triggered by glucose is not due to an osmotic effect, since mannitol was unable to mimic the glucose effect either on ABA content in the embryos or on *Rab16A* expression.

Sugar repression in higher plants, besides transcriptional regulation, involves modulation of the mRNA stability. Chen and Yu (1998) showed that the stability of mRNA is a critical factor related to sugar regulation. Analysis of the nucleotide sequences of rice α-amylase gene, *αAmy3* (also known as *RAmy3D*) showed that the stretch of a 9-bp AU-rich conserved sequence might be related to the stability of the mRNA. Glucose negatively affected the stability of the *Rab16A* mRNA in barley embryos (Figure 3-7), and further detailed analysis will be required to reveal the *cis*-acting sequences having sugar-response for mRNA stability.

Sugar repression of the β-Phaseolin gene of tobacco seed storage protein has been recently reported [Bustos et al. 1998]. Expression of the β-Phaseolin promoter-GUS (*PHSβ-uidA*) was induced by ABA, and is also modulated by sucrose and Ca^{2+}. The presence of sucrose in the medium inhibited the expression of *PHSβ-uidA* gene. However, the presence of both sucrose and Ca^{2+} restored the expression to 20-40 % of the maximum level measured in sucrose- and Ca^{2+}-free media [Bustos et al. 1998]. These results suggested that Ca^{2+} could revert the sugar effects. Experiment performed using barley embryos revealed that the *Rab16A* mRNA level decreases when glucose was added to the medium, but the presence of Ca^{2+} in the medium did not alter the expression pattern of *Rab16A* even in the presence of increasing glucose concentrations (data not shown). It is

possible that Ca^{2+} signaling is not directly involved in the cross-talk between the sugar and ABA signal transduction pathways as far as cereal embryos are concerned.

Glucose regulation of endogenous ABA level in cereal embryos

Sugars affect GA synthesis in barley embryos [Perata et al. 1997, Radley 1967]. Perata et al. (1997) reported that incubation of barley embryos in 25 mM glucose reduces the endogenous ABA level. My data indicate that the cellular sugar status affects the endogenous ABA level also in the rice embryos (Figure 3-1). However, glucose feeding to ABA-treated barley embryos did not affect the endogenous ABA content (Figure 3-6B), and similar results were also obtained when using rice embryos (data not shown). The ABA content in barley embryos after treatment with 10 μM ABA, either in the presence or absence of glucose or mannitol, was more than 100-fold higher than that measured in control embryos (Figure 3-6B), suggesting that glucose does not affect the ABA level when ABA is exogenously fed to the plant material.

Glucose and ABA effects on the expression of Rab16A *and* RAmy3D *gene*

The product of *Rab16A* gene belongs to the group 2 Lea protein family, also called dehydrins [Hattori and Hobo 1999]. It is assumed that the function of Lea proteins such as dehydrins is to protect cellular components from osmotic stress [Dure et al. 1989]. The promoter region of the *Rab16A* gene has two highly conserved motifs, motif I and motif II [Yamaguchi-Shinozaki et al. 1989]. Motif I (cgtACGTggc), containing the ACGT core sequence, is an essential part of the ABA-responsive *cis*-acting element, ABRE [Skriver et al. 1991]. The ACGT core sequence is also identified as a consensus sequence of the G motif (cctACGTggc) of the rice α-amylase (*RAmy3D*) gene promoter, which is the responsible *cis*-acting sequence for glucose repression [Hwang et al. 1998, Lu et al. 1998, Toyofuku et al. 1998]. Therefore, the ACGT core sequence might be a consensus sequence for

ABA-response as well as for glucose-response.

The promoter activity of the rice α-amylase gene *RAmy3D* was repressed by glucose (Figure 3-3B). My experiments revealed that glucose depletion results in an increased ABA content in the rice embryos (Figure 3-1), suggesting that ABA might be involved in the glucose repression of the *RAmy3D* gene. However, transient expression experiment using the *RAmy3D* promoter-*GUS* construct and Northern analysis revealed that glucose repression of the promoter activity of the *RAmy3D* gene is not affected by ABA or fluridone (Figure 3-3). These results indicate that the increase in endogenous ABA level triggered by glucose depletion does not affect the promotion of *RAmy3D* transcription.

The G motif sequence is considered to reside in the promoters of many genes that are switched on in response to diverse stimulatory pathways (i.e. light, anaerobiosis and phytohormones such as ABA). Indeed, G motif sequences in the promoter of the barley ABA-responsive *HVA22* gene are involved in ABA response [Shen and Ho 1995]. Further studies defined modular nature of the abscisic acid response complex (ABRC), the promoter unit necessary and sufficient for ABA induction of gene expression in barley [Shen et al. 1996]. *Rab16A* gene also has a G motif sequence in the promoter region known as motif I, which is an essential part of the ABA-responsive *cis*-acting element, ABRE. Nakagawa *et al.* (1996) reported that the *OSBZ8* gene encoded a basic region leucine zipper (bZIP) protein which is bound to the G motif and ABREs in the promoters of ABA-inducible genes, *Osem* and *Rab16A*. The transcripts of *OSBZ8* gene accumulate in response to ABA prior to accumulation of *Osem* and *Rab16A* mRNAs. These results suggested that *OSBZ8* might be involved in the regulation of transcription induced by ABA in rice. Although both G motifs of *RAmy3D* and *Rab16A* gene posses the conservative core sequence (ACGT), variable border sequence might be responsible for distinct response to ABA. The putative *trans*-acting factor(s) responsive to glucose signaling may bind to both the G motif sequences of *RAmy3D* and *Rab16A* genes to repress their transcription, but the factor(s)

responding to ABA, like OSBZ8, are able to bind to the G motif sequence in the promoter region of *Rab16A* gene to promote its transcription, but not to that in the *RAmy3D* gene. Indeed, a bZIP transcription factor in *Arabidopsis* was reported to be involved in glucose signaling process(es) [Rook et al. 1998]. Further experiment will be needed to examine this hypothesis.

3. 5 Summary

Glucose effects on the expression of the abscisic acid-inducible *Rab16A* gene were examined using rice and barley embryos. Glucose feeding to rice embryos negatively affects the endogenous abscisic acid content and represses the promoter activity of the *Rab16A* gene. Glucose repression of the *Rab16A* gene takes place both at transcriptional and post-transcriptional level. Modulation of the abscisic acid content in the rice embryos triggered by glucose did not directly influence the expression of the rice α-amylase gene *RAmy3D*, which is known to be under glucose control. The possible interaction between the glucose and abscisic acid signaling pathway was discussed.

3. 6 References

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Figures

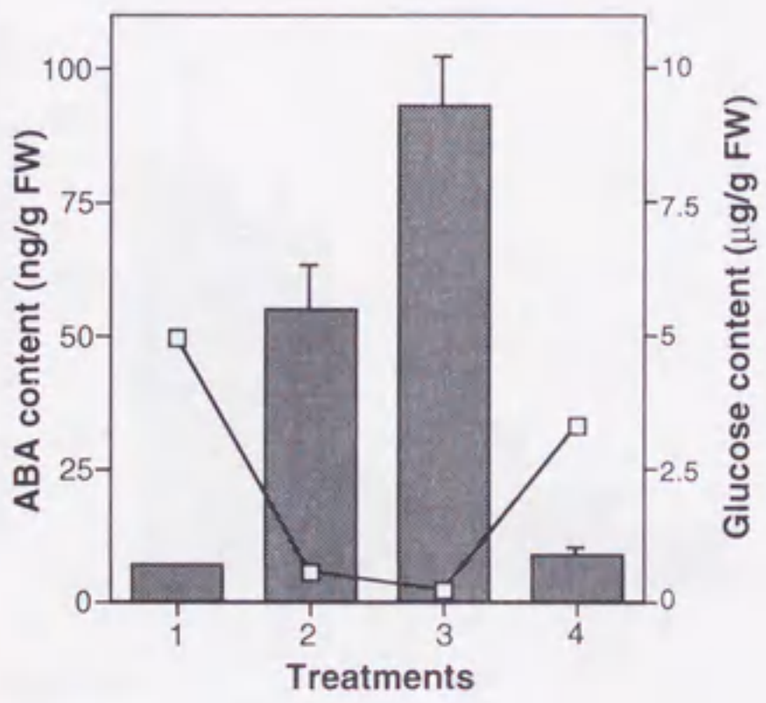


Figure 3-1 Effect of glucose treatment and sugar depletion on the endogenous ABA and glucose content of callus-forming rice embryos.

Callus-forming rice embryos analyzed immediately after excision from the endosperm (Treatment 1), as well as after 1- and 3-d incubation of the excised embryos on sugar-free medium (i.e. glucose depletion; Treatments 2 and 3) and on medium containing 90 mM glucose for 2 days after 1-d glucose starvation (Treatment 4). ABA content was quantified by using a monoclonal antibody (see Materials and Methods). Glucose content (❑) was estimated by enzyme coupling assay [Guglielminetti et al. 1995]. FW, fresh weight.

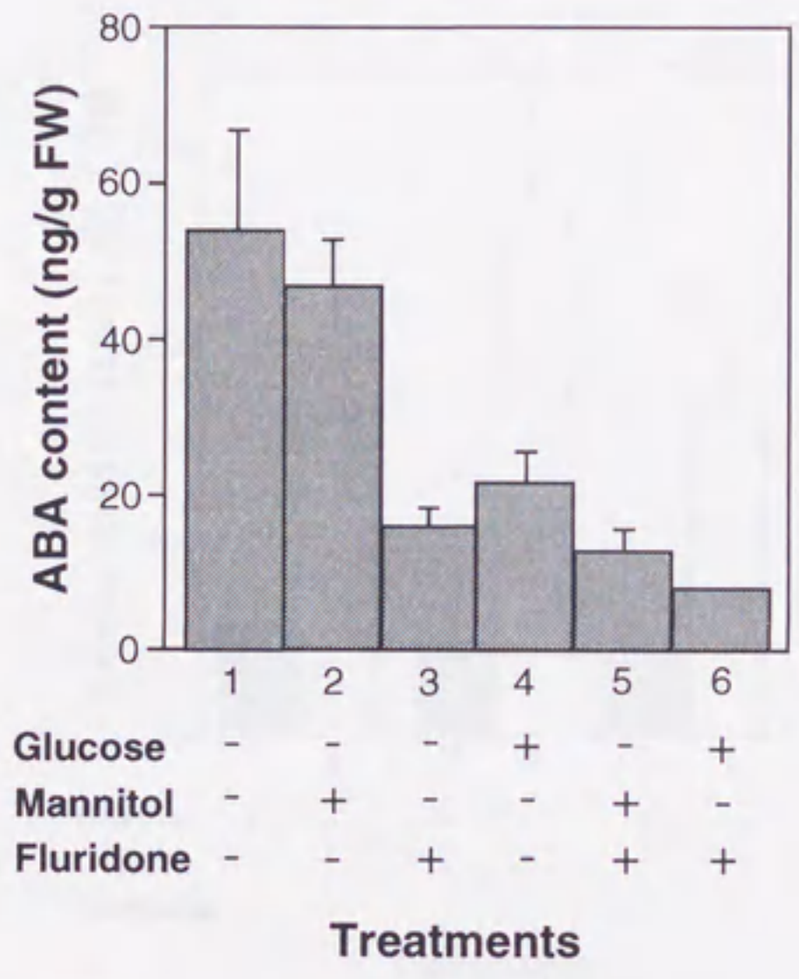


Figure 3-2 Effect of glucose and fluridone on the endogenous ABA content of rice embryos.

After excision of callus-forming rice embryos from the endosperm, they were incubated (1 day) in petri dishes containing Murashige-Skoog salt mixture and 2 mg/l 2,4-D with or without 90 mM glucose, 90 mM mannitol, 300 µM fluridone. ABA content was quantified by using a monoclonal antibody.

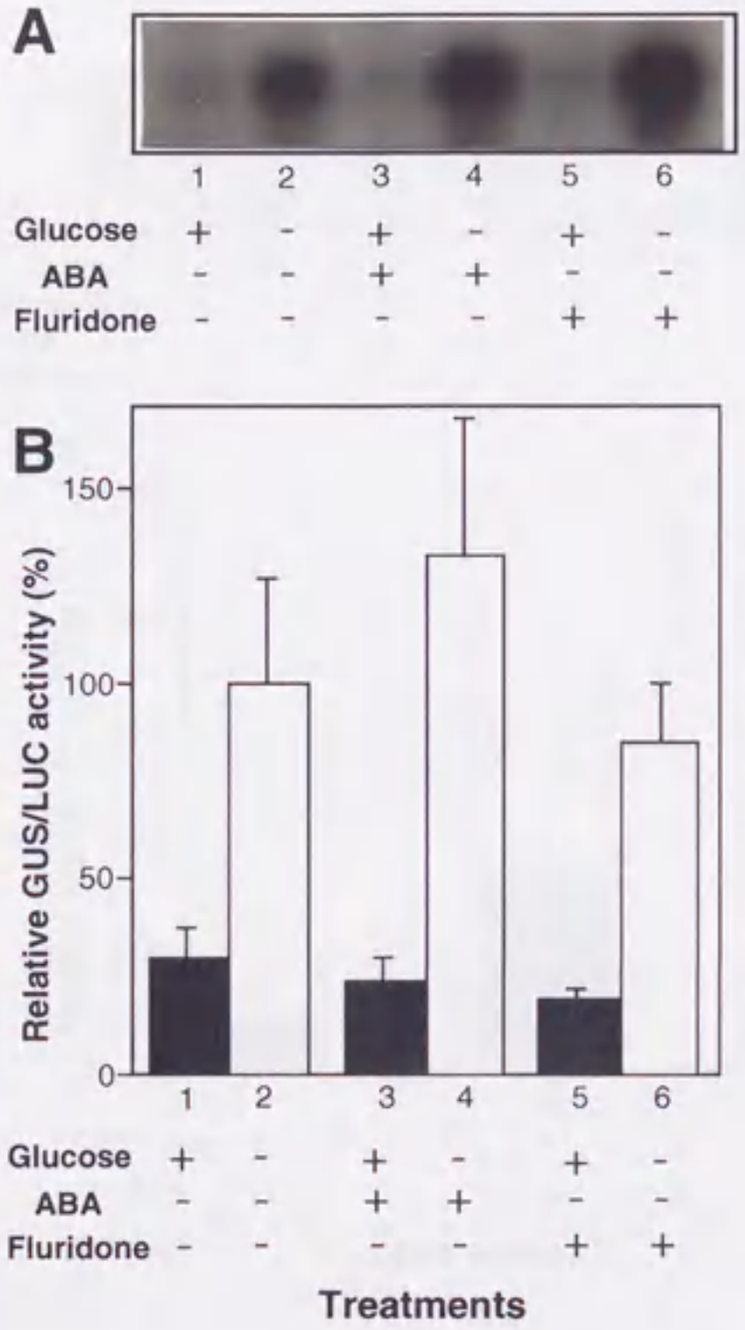


Figure 3-3 Effect of glucose, ABA and fluridone on *RAmy3D* gene expression in rice embryos.

A: Effect of glucose, ABA and fluridone on mRNA level of the rice α-amylase *RAmy3D* gene by Northern blot.
After excision of callus-forming rice embryos from the endosperm, they were incubated (1 day) in petri dishes containing Murashige-Skoog salt mixture and 2 mg/l 2,4-D with or without 90 mM glucose, 10 μM ABA and 300 μM fluridone.
B: Effect of glucose, ABA and fluridone on promoter activity of the rice α-amylase gene *RAmy3D* using the transient expression system.
After bombardment, embryos were treated as in panel **A**.
RAmy3D-GUS expression data were normalized by using the *35S-LUC* construct as an internal standard. Relative GUS/LUC activity is expressed as: control (Treatment 2)=100. Data are mean±SE.

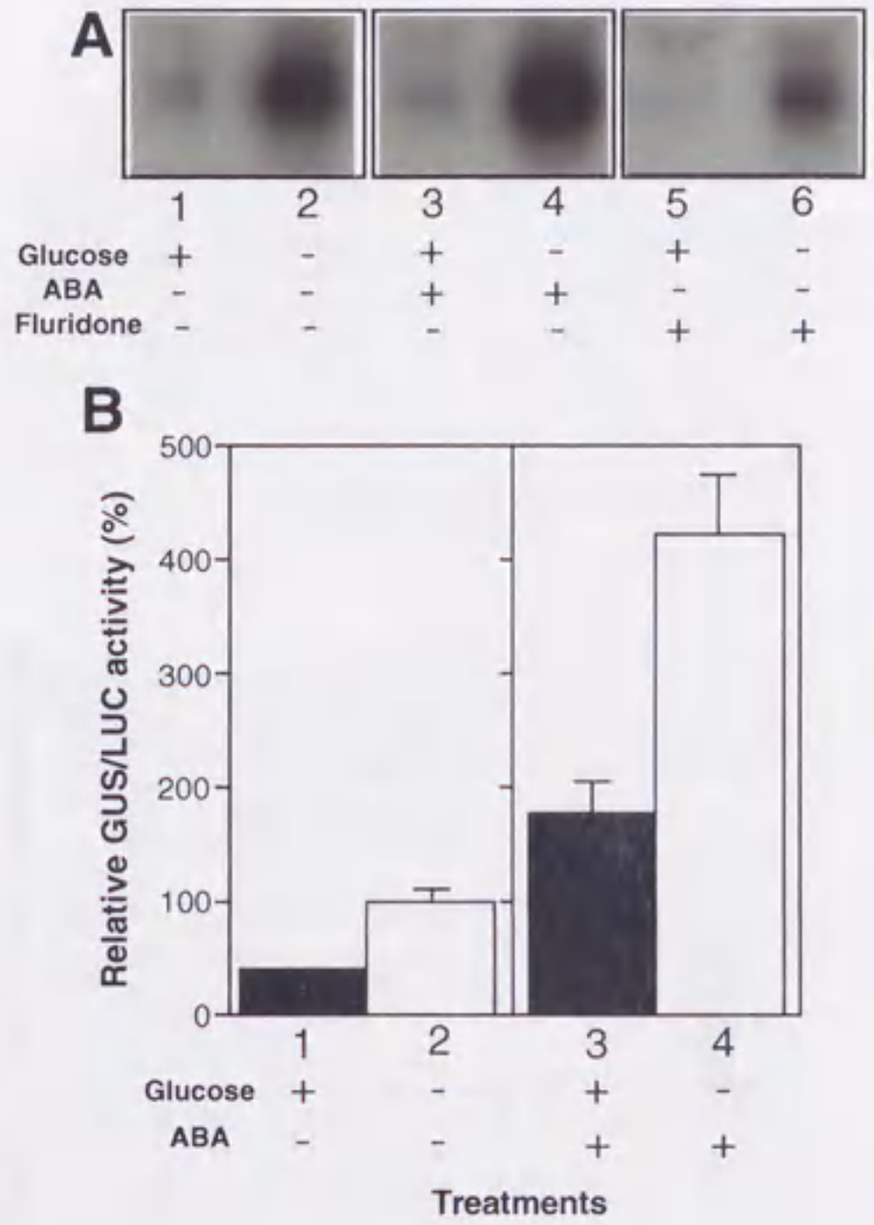
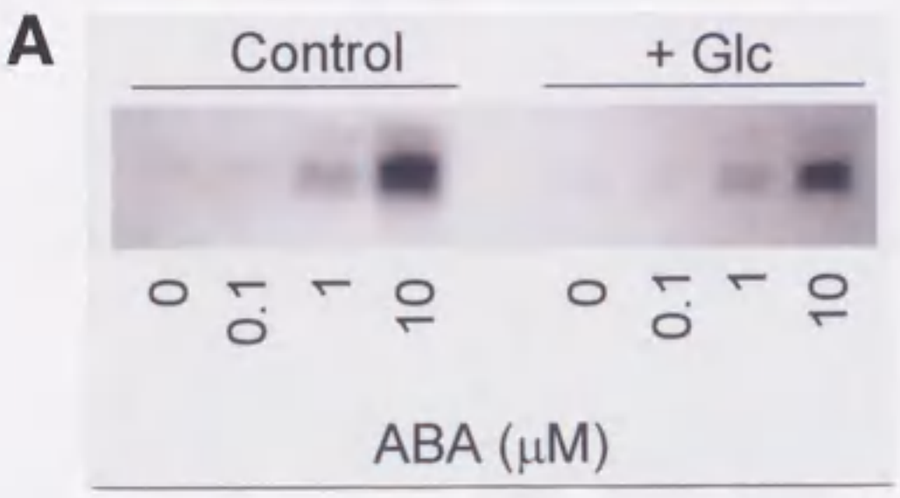


Figure 3-4 Effect of glucose, ABA and fluridone on *Rab16A* gene expression in rice embryos.

A: Effect of glucose, ABA and fluridone on *Rab16A* mRNA level.
After excision of callus-forming rice embryos from the endosperm, they were incubated (1 day) in petri dishes containing Murashige-Skoog salt mixture and 2 mg/l 2,4-D with or without 90 mM glucose, 10 μM ABA and 300 μM fluridone. A representative RNA gel blot probed with the *Rab16A* probe is reported.
B: Effect of glucose and ABA on *Rab16A* promoter activity.
After bombardment, embryos were incubated (1 day) in petri dishes containing Murashige-Skoog salt mixture and 2 mg/l 2,4-D with or without 90 mM glucose, and 10 μM ABA.
Rab16A-GUS expression in data was normalized by using the *35S-LUC* construct as an internal standard. Relative GUS/LUC activity is expressed as: control (Treatment 2; no glucose and ABA)=100. Data are mean±SE.



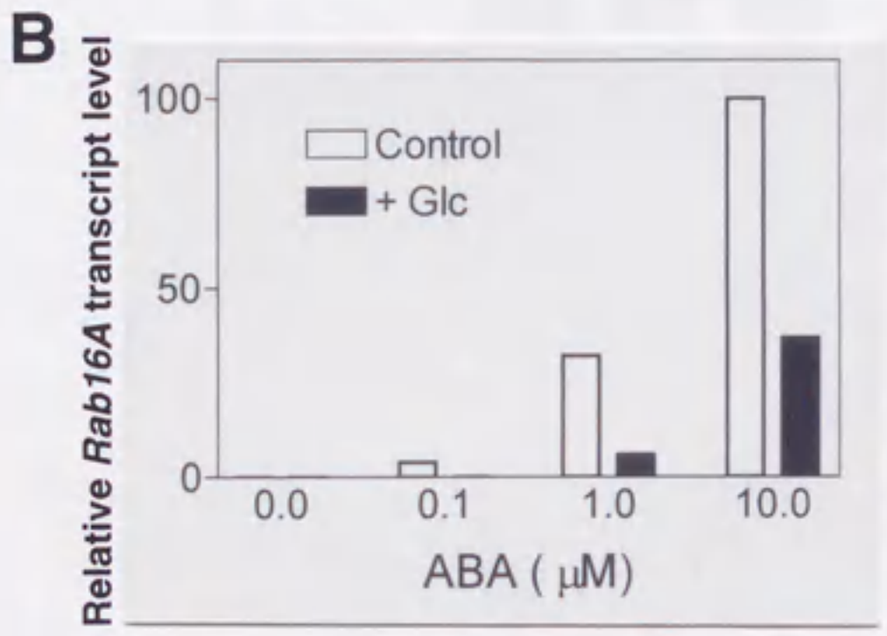


Figure 3-5 Effect of ABA on *Rab16A* mRNA in barley embryos.

Barley embryos were dissected from dry barley grains and incubated in a medium containing increasing ABA concentrations in the presence or absence of glucose (50 mM). Panel **A** shows the RNA gel blot probed with the *Rab16A* probe, while panel **B** reports the densitometric quantitation of the RNA blot after correction for RNA loading on the gel by densitometric quantitation of the same RNA blot probed with a rRNA probe. The relative transcript level of 100 is assigned to the higher level of transcript detected in the experiment.

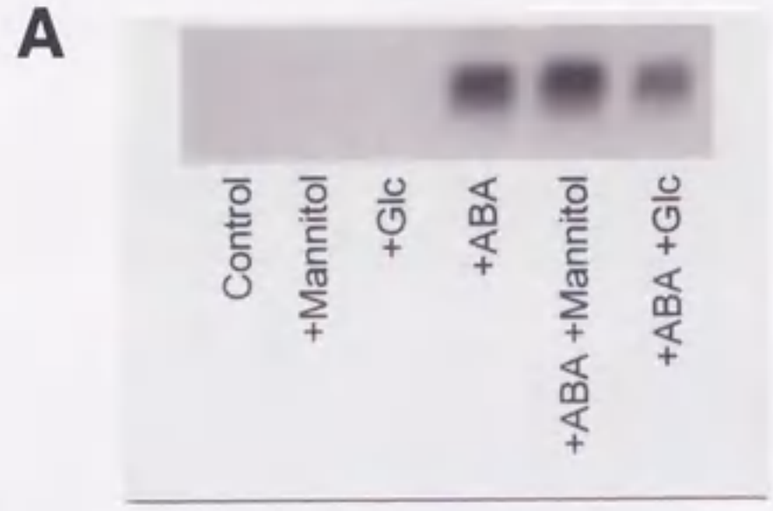


Figure 3-6 Effects of glucose on ABA levels in barley.

Barley embryos were dissected from dry barley grains and incubated in a medium containing the compounds indicated in the Figure 3-4. Concentration of ABA, glucose and mannitol was 10 µM, 50 mM and 50 mM, respectively.
Panel **A** reports the RNA gel blot probed with the *Rab16A* probe; equal loading was verified by densitometric quantitation of the same RNA blot probed with a rRNA probe (not shown). Panel **B** reports the ABA quantitation in the embryos incubated without (Treatment 1) and with 10 µM ABA (Treatment 2) in the presence of 50 mM mannitol (Treatment 3) and glucose (Treatment 4). ABA data are mean (±SE) of 8 replicate experiments, each including 4 embryos.

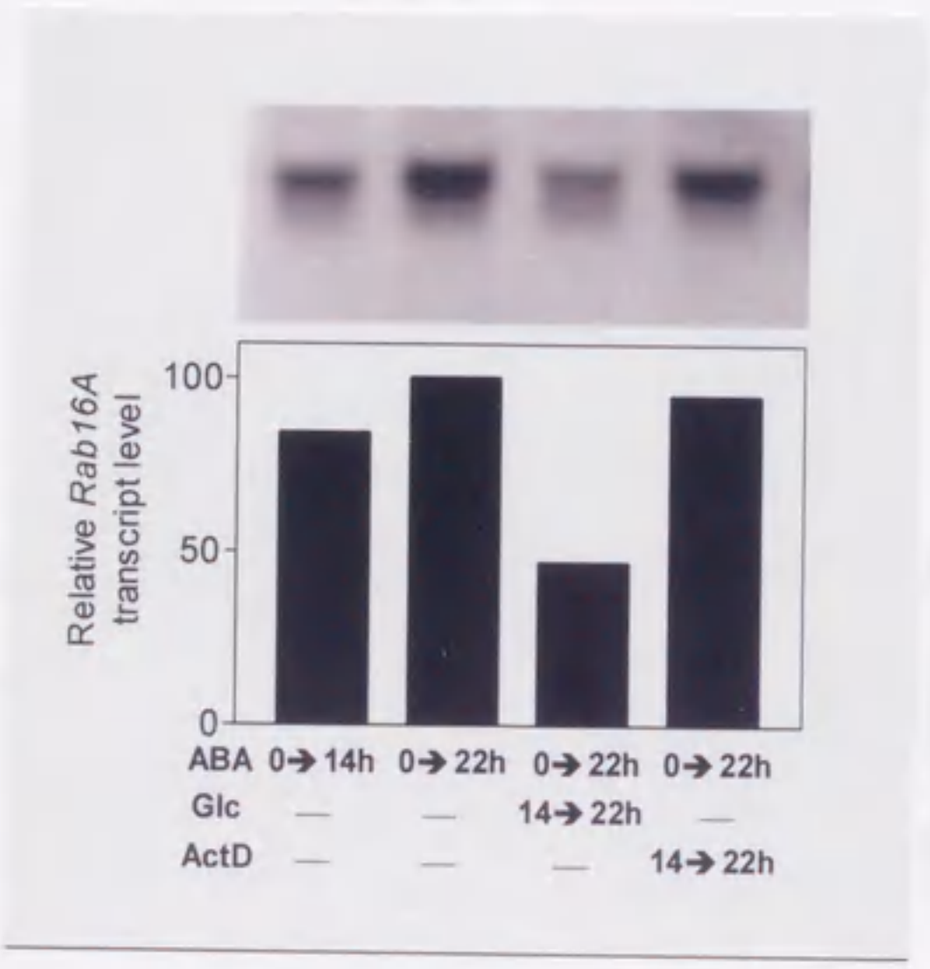


Figure 3-7 Effect of glucose on *Rab16A* mRNA stability.

Barley embryos were dissected from dry barley grains and incubated in a medium containing 10 µM ABA for 14-22 h. Glucose (50 mM) or actinomycin-D (ActD, 10 µg/ml) were added after 14 h and incubation proceeded for further 8 h (up to 22 h). Upper panel shows the RNA gel blot probed with the *Rab16A* probe, while the lower panel reports the densitometric quantitation of the RNA blot after correction for RNA loading on the gel by densitometric quantitation of the same RNA blot probed with a rRNA probe. The relative transcript level of 100 is assigned to the higher level of transcript detected in the experiment.

Chapter 4

Characterization and expression of monosaccharide transporters (*OsMSTs*) in rice

4. 1 Introduction

Although green plants are carbon autotrophic organisms that synthesize their sugars as well as all other organic compounds photosynthetically, plant cells nevertheless require sugar-uptake mechanisms. Higher plants are physiological mosaics of autotrophic green and heterotrophic non-green cells and tissues. The roots, stems, and reproductive organs are supplied with organic compounds, and the leaves deliver sucrose and other oligosaccharides to them. The transporter which putatively catalyzes the import of sugars has been cloned. The first plant glucose transporter was isolated from *Chlorella kessleri* [Sauer and Tanner 1989], and the sucrose transporter was isolated from spinach in 1992 [Riesmeier et al. 1992]. These discoveries facilitated the attempt to clarify the plant sugar transport system.

In 'source' tissue in higher plants, carbohydrates are produced by photosynthesis or starch degradation. After sucrose synthesis by photosynthetic CO_2 fixation in green leaves or glucose production by starch degradation, these soluble carbohydrates have to transport to 'sink' tissue in order to consume carbohydrates for plant development or storage. Assimilated carbon is loaded into phloem cells symplastically and/or apoplastically to photosynthetically inactive sink organs [van Bel 1993]. Symplastic transport of sucrose depends on the existence of symplastic connections such as plasmodesmata, while the apoplastic phloem loading needs an energy-dependent active transport system, i.e., a sucrose transporter.

In 'sink' tissue, hexoses produced through hydrolysis of the unloaded sucrose by apoplastic invertase are taken up into cells by a hexose (monosaccharide) transporter in addition to the direct uptake of sucrose by a sucrose transporter. The monosaccharide tranaporter can be identified up to 26 genes in *Arabidopsis* [Lalonde et al. 1999] and about 20 genes in yeast [Kruckeberg 1996], making a large gene family. Studies on plant

monosaccharide transporters suggested that the transporters mainly play a role on hexose uptake in sink tissue [Weber et al. 1997, Sauer and Stadler 1993] and the allocation of sugar in response to various environmental stresses such as mechanical wounding or pathogen attack [Truernit et al. 1996].

In this Chapter I describes the first cloning of full-length cDNA clones of the monosaccharide transporter gene from rice and the characterization of the corresponding gene products by heterologous expression in yeast *S. cerevisiae*. Furthermore, I have examined their cell-specific expression in root by an *in situ* mRNA detection technique.

4. 2 Materials and methods

OsMST *clones*

OsMST1-3 cDNAs (EST accession number: D25142, D46606, D40232, respectively) were obtained from the Rice Genome Research Program. The cDNA sequence was determined by the dideoxy-chain termination method using an ABI 373A DNA sequencer (Perkin-Elmer Co.).

DNA extraction and Southern blot analysis

For Southern blot analysis, the genomic DNA of rice (Japonica cultivar: Nipponbare) was extracted from green-leaf tissue by an ISOPLANT DNA isolation kit (Nippon GENE Co., Japan). Genomic DNA (2 μg) was digested with restriction enzymes, electrophoresed on 0.7 % agarose gel, and blotted onto a nylon membrane (Hybond N^+; Amersham) under alkaline conditions. Membranes were prehybridized for 1 h at 65 °C in 5x Denhardt's solution (1x Denhardt's solution: 0.1 % bovine serum albumin, Ficoll 400, and polivinyl pyrrolidone), 0.5 % SDS, 50 % formamide, 5x SSPE (1x SSPE: 0.15 M NaCl, 8.65 mM NaH_2PO_4, and 1.25 mM EDTA), and 50 μg/ml salmon sperm DNA. Radiolabeled probes were prepared from gel-purified cDNA fragments (*OsMST1*, 1100 bp; *2*, 820; *3*, 870) which were the same as those used in Northern blot analysis by random primer labeling with α-^{32}P-dCTP. Hybridization was performed at 65 °C in the same buffer to that of the prehybridization. The membranes were washed once in 2x SSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 1 % SDS for 15 min and twice in 0.2x SSC and 1 % SDS at 15-min intervals at 65 °C. Membranes were exposed using a Fujix BAS2000 Bio-Imaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

RNA extraction and Northern blot analysis

RNA extraction from isolated dry embryo; leaf sheath, leaf blade, etiolated

shoot and root of 10-d-old plants was performed by using the aurintricarboxylic acid method, as described by Skadsen (1993), with minor modifications. Total RNA (15 μg) of each sample was electrophoresed on formaldehyde gel and blotted onto a nylon membrane (Hybond N^+; Amersham). The subsequent hybridization and washing processes were conducted by the same conditions to those of the Southern blot analysis at 42 °C for hybridization and washing.

Sugar uptake by heterologously expressed yeast

The construction of plasmids for expression of *OsMSTs* in Saccharomyces cerevisiae was achieved by using a *GAL* expression system in a multicopy plasmid pTV3e [Nishizawa et al. 1995]. The open reading frame (ORF) of *OsMST1-3* was replaced to that of *GAL2* in a pTV3e cassette vector by *Eco*RI and *Cla*I sites. Plasmids were introduced into LBY416 (*MATα hxt2::LEU2 snf3::HIS3 gal2 lys2 ade2 trp1 his3 leu2 ura3*) [Nishizawa et al. 1995]. Transport of glucose and other monosaccharides in yeast cells was performed by the procedures described previously [Kasahara et al. 1997]. Briefly, the transport assay medium used was 50 mM MES-NaOH (pH 6.0) containing 2 mM $MgSO_4$, and the transport reaction was stopped using assay medium containing 0.5 mM $HgCl_2$. The initial rate of glucose transport was assessed by the transport of 0.1 mM D-[U-^{14}C] glucose (CFB96; Amersham) or D-[U-^{14}C] xylose (CFB59; Amersham) or 3-*O*-methyl-D-[U-^{14}C] glucose (CFB141; Amersham) for 5 s-25 min by experiment, at 30 °C.

In situ *hybridization*

Isolated rice roots were fixed in FAA (formalin-acetic acid-50 % ethanol [1:1:18]) for 48 hr at 4 °C. After dehydration in a graded 2-methyl-2-propanol series, samples were embedded in paraplast (Oxford Labware, St. Louis, MO) and sectioned at 10 μm by rotary microtome, and then applied on slide glasses treated with 3-aminopropyltrichlorosilane (Shinetsu Chemicals, Tokyo, Japan). A digoxygenin-labeled RNA probe of rice (*OsMST1*, 1300 bp; *2*; 820; *3*, 880) was

prepared from the coding region of an *OsMST1-3* cDNA clone, which was basically the same position as those used in Northern and Southern blot analyses. Probes were degraded to a mean length of 150 bp by incubating in alkali at 60 °C. *In situ* hybridization was performed according to Kouchi and Hata (1993). Hybridization signals were also detected according to Kouchi and Hata (1993). No hybridization signal was detected when sense probes were used.

4. 3 Results

Monosaccharide transporter in rice

Comparison of the putative amino acid sequences among OsMST1-3 showed 31.5 to 57.7 % homology to each other (Figure 4-1). The OsMST1, 2 and 3 proteins have a length of 517, 522 and 518 amino acids, respectively, and a calculated molecular weight of 56.9, 57.4 and 57.0 kDa, respectively. The hydrophobicity profile revealed that the OsMST1-3 has putative twelve transmembrane domains and two sets of six transmembrane domains separated by a central long hydrophilic region (Figure 4-2). This topological pattern was consistent with those of sugar transporters in microbes, mammals, and plants [Henderson et al. 1992].

Genomic Southern blot analysis was conducted to estimate the number of genes coding for monosaccharide transporter in rice genome. Rice genomic DNA was digested with five different restriction enzymes (*Eco*RI, *Hin*dIII, *Bam*HI, *Eco*RV, and *Bgl*II) and hybridized with gel-purified cDNA fragments which were the same as those used in Northern blot analysis. There was no restriction site for the enzymes used in this DNA probes except for *Hin*dIII (one site in *OsMST2*). For *OsMST1*, only a major single band in all digestions and one faint band in *Eco*RI were detectable, while one major and some minor bands were detectable for *OsMST2* and *3* in all the digestions (Figure 4-3). These data suggested that at least several and possibly ten or more copies of the genes encoded for monosaccharide transporter and related genes exist in the rice genome.

Sequence alignment compared with rice and others

The phylogenetic tree based on the deduced amino acid sequences of monosaccharide transporters among human, yeast, and plant is shown in Figure 4-4. GULT1 (human) and RGT2, HXT2 and GAL2 (yeast) are diverse from others. The RGT2 protein is a unusual glucose transporter and serves as glucose sensor in

the membrane that generate an intracellular glucose signal, therefore the transport activity is hardly detectable. No significant diversity was found among the plant transporters from monocots to dicots, and OsMST1 had a slight phylogenetic distance in comparison with OsMST2 and 3 (Figure 4-4).

The motifs from the deduced amino acid sequences of the monosaccharide transport were highly conserved among rice (*OsMST1-3)*, castor bean (*HXT6*), *Arabidopsis* (*MST1*), and human (*GLUT1*), with a similar position of twelve hydrophobic transmembrane domains separated by a central long hydrophilic region (Figure 4-1). Henderson et al. (1992) and Pao et al. (1998) proposed conserved motifs on sugar transport proteins including those having transport activity of monosaccharides. As shown in Figure 4-1, the amino acid residues indicated by asterisks, which were reported by Henderson et al. (1992) as motifs related to the sugar transport, are highly conserved among the OsMST1-3 proteins.

Functional expression of the OsMST proteins in yeast

To verify whether OsMST1-3 proteins could function as monosaccharide transporters, cDNAs were subcloned in a *GAL* expression vector and introduced into LBY416, a strain of *S. cerevisiae* in which high-affinity glucose transport activity is kept low, since three monosaccharide transporter-related genes, *HXT2, GAL2* and *SNF3*, have been disrupted. Subcloned cDNAs were expressed under control of the *GAL2* promoter in the presence of galactose [Nishizawa et al. 1995]. Addition of $HgCl_2$ as an SH-group inhibitor completely abolished the transport activity (Figure 4-5). The cell with an empty vector (control) showed a low level of glucose transport in the absence of $HgCl_2$, which identified the low activity of the cell caused by minor sugar transporter(s) as a background. While OsMST1 did not show any activity, the glucose transport activity of OsMST2 and 3 was detected as nearly two and three times higher compared with that of the control, respectively (Figure 4-5). Since the OsMST3 protein had the most intense activity, I used OsMST3 for the further characterization.

Substrate specificity and energization of sugar uptake of the OsMST3 proteins

In order to analyze further transport characteristics of OsMST3, experiments using distinct monosaccharides as the substrate were tested. To estimate the energy-dependent active transport system or facilitating system of OsMST3, D-glucose, D-xylose, and 3-*O*-metyl glucose (3-OMG), the classical non-metabolizable substrate analog for D-glucose, were used as transport substrates. Compared with D-xylose (Figure 4-6B) and 3-OMG (data not shown), D-glucose showed the highest transport activity (Figure 4-6A), at 10 fold and 7 fold.

The uptake level of D-xylose in pTV3e did not change at a considerably low level through the tracing period (5 min) whether ethanol was added to the cells or not (Figure 4-6C). This was consistent with the result that *S. cerevisiae* cells are unable to uptake and metabolize D-xylose [Kötter et al., 1990]. Energization uptaken by added ethanol occurred in all monosaccharides tested in the transport experiments (D-glucose, D-xylose, and 3-OMG) (Figure 4-6). Ethanol seems to serve as an electron donor of the electrogenic transmembrane transport of monosaccharides when the applied substrate cannot be metabolized by yeast itself [Sauer and Stadler, 1993].

D-xylose was used for further investigation of the energy dependency of the transport of OsMST3. This is a good substrate for this experiment because, when the yeast strain LBY416 could not uptake D-xylose (Figure 4-6C), the transport background of yeast cells was low. Figures 4-6B and 7 show that the addition of ethanol caused a rapid D-xylose influx into the cell with OsMST3. This energization on the plasma membrane of yeast cells was reversible when uncoupler carbonyl-cyanide-*m*-chlorophenyl-hydrazone (CCCP) was applied (Figure 4-7). These results suggested that D-xylose accumulated in the cells is caused to efflux rapidly out of the cell as a result of the plasma membrane being de-energized by the CCCP. Because of the acceleration by energization and the inhibition after addition of the uncoupler, the OsMST3 protein serves as an energy-dependent

monosaccharide transport system, possibly an H^+ symporter.

Cell-specific expression of OsMST

Northern blot analysis using total RNA from various rice tissues was conducted to identify where *OsMST* mRNAs are detectable. Weak signals for *OsMST2* and *3* were detected in leaf sheath, leaf blade, and root, but no signals were detected for *OsMST1* (Figure 4-8). To examine the strict location of cells, *in situ* hybridization using *OsMST1-3* antisense probes was performed, indicating that both *OsMST2* and *3* are exclusively expressed in sclerenchyma and xylem cells (Figure 4-9). No signals were detectable for *OsMST1*, which coincided with the result by Northern blot analysis. The cells in which *OsMST2* and *3* were expressed seem to be distinctly characteristic of the cell wall; namely, they are thickened cells having lignified, secondary walls.

4. 4 Discussion

OsMST2 and 3 as monosaccharide transporter in rice

The amino acid sequences and the topological pattern of the OsMST1-3 proteins, rice monosaccharide transporters were highly homologous to other monosaccharide transporters in plants previously identified (Figures 4-1, 2 and 4), but the OsMST1 protein was the only one that did not show any transport activity (Figure 4-5).

As shown in Figures 4-6 and 7, the OsMST3 are energy-dependent active transporters similar to those of many other plant monosaccharide transporters reported so far [Caspari et al. 1994, 1996, Weig et al. 1994]. When D-xylose or non-metabolizable glucose analog, 3-OMG, was used as transport substrate, yeast cells possessing OsMST3 could transport them. In D-xylose, the transport rate increased up to about 4.7 fold compared with that with a vector, and addition of ethanol resulted in a further increase of transport of D-xylose to about 6.7 fold. The heterologous expression also demonstrated that the characteristics, such as sensitivity to uncoupler and blocking of transport by SH-group inhibitor, point to OsMST3 protein as an H^+ symporter (Figures 4-6 and 7). According to the classification of transporter in living cells by Pao et al. (1998), OsMST3 protein can be classified into SP (sugar porter) family in MFS (major facilitator superfamily), also called the uniporter-symporter-antiporter family.

Buckhout (1989) and Bush (1990) reported that the presence of a pH gradient (ΔpH) across the vesicle membrane and the membrane potential ($\Delta\psi$) is significantly related to proton-sucrose symport of plasma membrane vesicles isolated from sugar beet. Energization of the plasma membrane transport by adding metabolizable substances such as ethanol as an electron donor was also observed in this report (Figures 4-6 and 7). Instances of negatively charged $\Delta\psi$ drive the sugar transport activity [Kalinin and Opritov 1989, Buckhout 1989, Bush 1990].

OsMST3 *shows specific expression in cells with a thickened cell wall*

As shown in Figure 4-8, the rice sucrose transporter, *OsSUT1* [Hirose et al. 1997] mRNA was expressed in callus and leaf sheath in the young stage. *In situ* hybridization analysis revealed that the expression of *OsSUT1* is exclusively localized in companion cells of the leaf sheath in rice [Matsukura et al., unpublished data]. It is likely that sucrose transporters play a key role in phloem loading of sucrose [Kühn et al. 1997, Schulz et al. 1998, Burkle et al. 1998].

In situ hybridization using *OsMST3* antisense probe revealed that the expression of *OsMST3* was highly restricted in the sclerenchyma and xylem cells in the young root (Figure 4-9). These cells have a distinct, specialized characteristic, namely, thickened cells with lignified, secondary walls. Sclerenchyma cells belong to the category of supporting cells, and cellulose is therefore positively synthesized in the cell wall during the immature stage to protect the vascular bundle from mechanical stresses. The role of the xylem is that of a water conductor by the tracheary element but may also serve as a kind of supporting tissue. Accordingly, both tissues have fundamentally the same character. It is commonly accepted that cellulose, the simple polymerization of glucose, is synthesized by UDP-glucose. These results suggested that OsMST3 serves to accumulate monosaccharides, being a substrate for the formation of cellulose when lignins are synthesized in the cell wall at the stage of cell thickness.

Sugar sensing by sugar transporters

During germination of cereal grains, α-amylase plays a key role in the mobilization of the energy reserves constituted by insoluble starch [Jones and Jacobsen 1991]. In rice, the modulation of α-amylase genes by carbohydrates and other metabolites is well described [Umemura et al. 1998]. The expression of *RAmy3D* gene is rigidly controlled by sugar, and the sugar-responsible elements of

this gene have been defined as the G motif and the GATA motif [Lu et al. 1998, Hwang et al. 1998, Toyofuku et al. 1998]. It is still unknown how the signal is transduced to activate or repress such a large number of sugar-sensitive genes and what kind of sensing mechanism is involved in the expression of those genes. A putative sugar sensor in plant cells may be hexokinase [Jang and Sheen 1997, Umemura et al. 1998]. In yeast, two unusual glucose transporters in the yeast appear to function as low- and high-glucose sensors, respectively, that generate an intracellular glucose signal [Özcan et al. 1996]. These sensors are required for maximum induction of several hexose transporter (*HXT*) genes, encoding glucose transporters, and this efficient system is needed for yeast and unicellular organisms to live and rapidly adapt to a variable environment. It may be speculated that similar sensor systems to those in yeast exist in multicellular organisms like higher plants, which consist of mosaics of autotrophic green and heterotrophic non-green cells. Because heterotrophic organs such as roots, stems, and reproductive tissues cannot produce carbohydrates themselves, autotrophic green organs which can produce carbohydrates by photosynthesis supply them. By reason of these morphogenically and physiologically complicated characteristics, great numbers of the transporter which catalyzes sugar transport might be involved in this process [Lalonde et al. 1999]. Genomic Southern blot analysis has shown a relatively large gene family for *OsMST* in this report (Figure 4-3). Further characterization will be required to clarify monosaccharide transport systems in plants.

4.5 Summary

This study examines the cloning and characterization of monosaccharide transporter cDNAs in rice. The OsMST1-3 (*Qryza sativa* monosaccharide transporter 1-3) has putative twelve hydrophobic transmembrane regions and two sets of six transmembrane domains separated by a central long hydrophilic region. Heterologous expression of the OsMST3 protein in yeast *S. cerevisiae* revealed that OsMST3 has transport activity for some monosaccharides and an energy-dependent H^+ co-transport manner. Northern blot and *in situ* hybridization analysis showed that *OsMST3* mRNA is detectable in leaf sheath, leaf blade, and root, especially the xylem as well as in sclerenchyma cells in root. These results suggested that OsMST3 is involved in the accumulation of monosaccharides required for cell wall synthesis at the stage of cell thickness.

4.6 References

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Figures

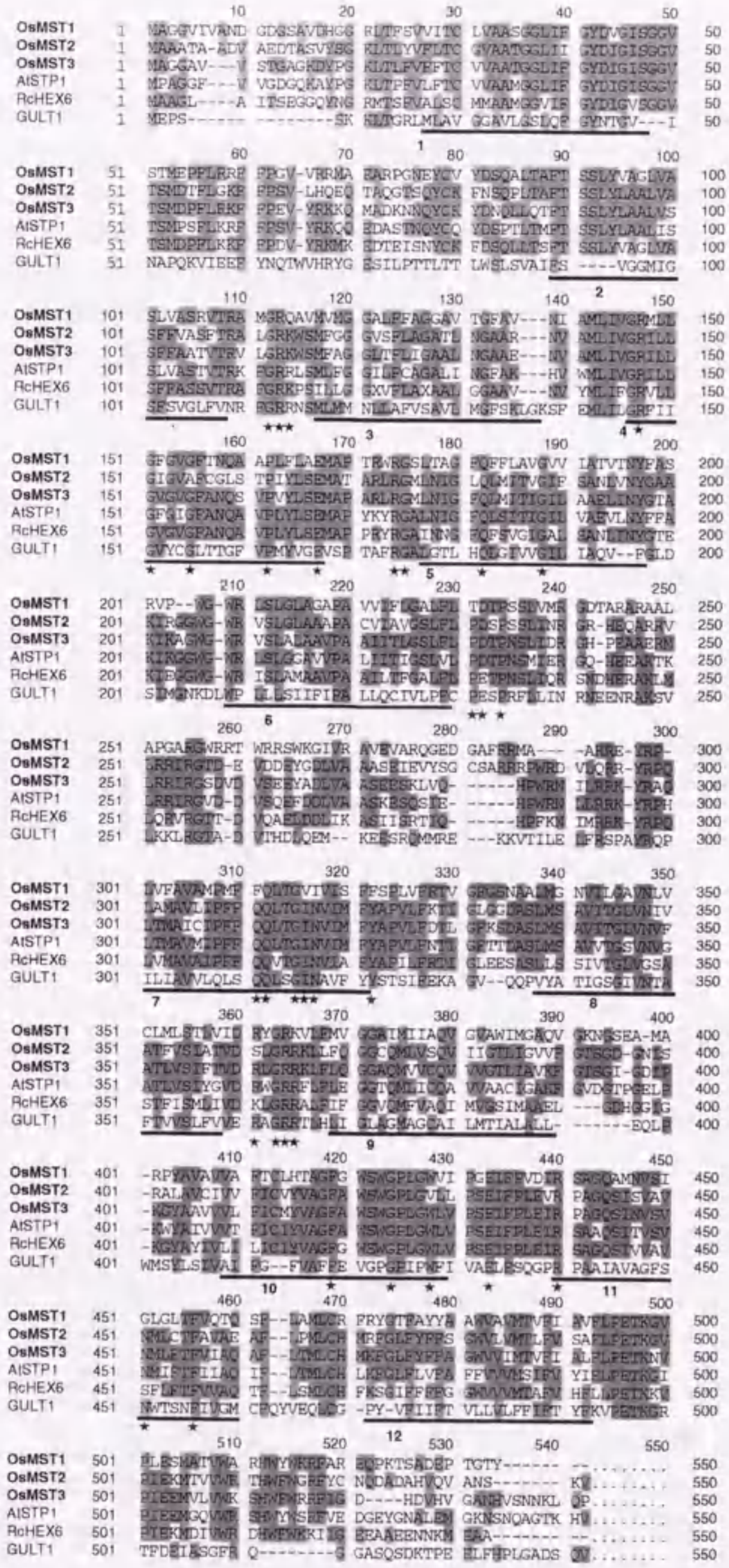
OsMST1
OsMST2
OsMST3
AtSTP1
RcHEX6
GULT1

Figure 4-1 The alignment of amino acid sequences of OsMST1-3 proteins with known monosaccharide transporters of other organisms.

The alignment of the predicted amino acid sequences of OsMST1-3 with those of AtSTP1 from *Arabidopsis thaliana* [Sauer et al. 1990], RcHEX6 from Caster bean [Weig et al. 1994], and GLUT1 from human [Mueckler et al. 1985] are shown. Black boxes indicate identical amino acid residues. Asterisks below the sequences indicate that the motifs [Henderson et al. 1992]. Putative transmembrane domains are underlined. Multiple-sequence alignment was constructed by DNASIS-Mac v.3.7.

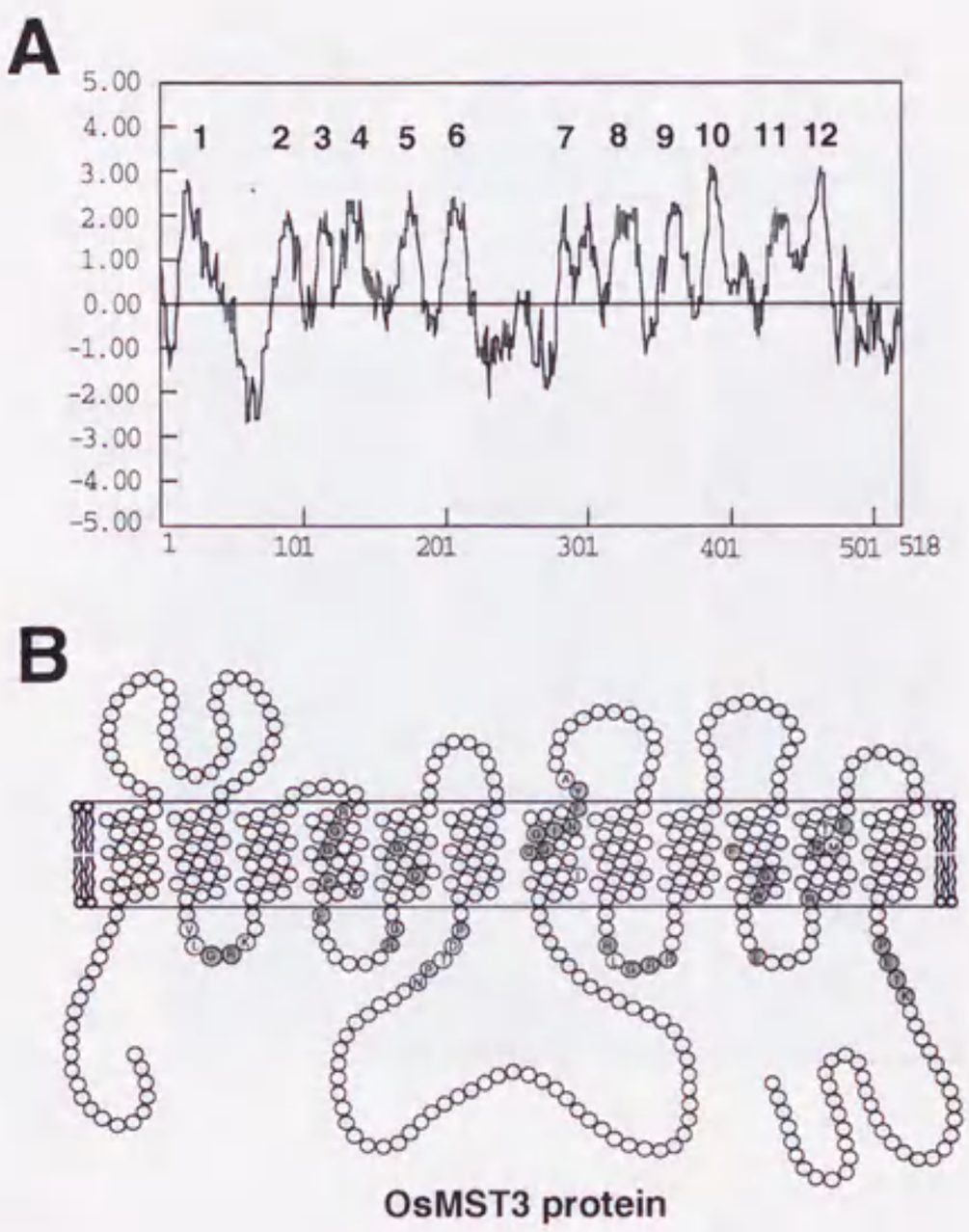

Figure 4-2 Hydropathy profile and a membrane-spanning model of OsMST3.

A: The calculation was done according to the algorithm of Kyte and Doolittle (1982). Numbers shown are the predicted transmembrane domains.

B: Schematic representation of the OsMST3 protein.

A twelve-transmembrane spanning model is based on Baldwin (1993). Letters inside the circles indicate the amino acid residues composing the motifs predicted to have a relation to the monosaccharide transport, and shaded circles indicate the conserved amino acid residues in the OsMST3 protein. Similar results were also obtained for OsMST1 and 2.

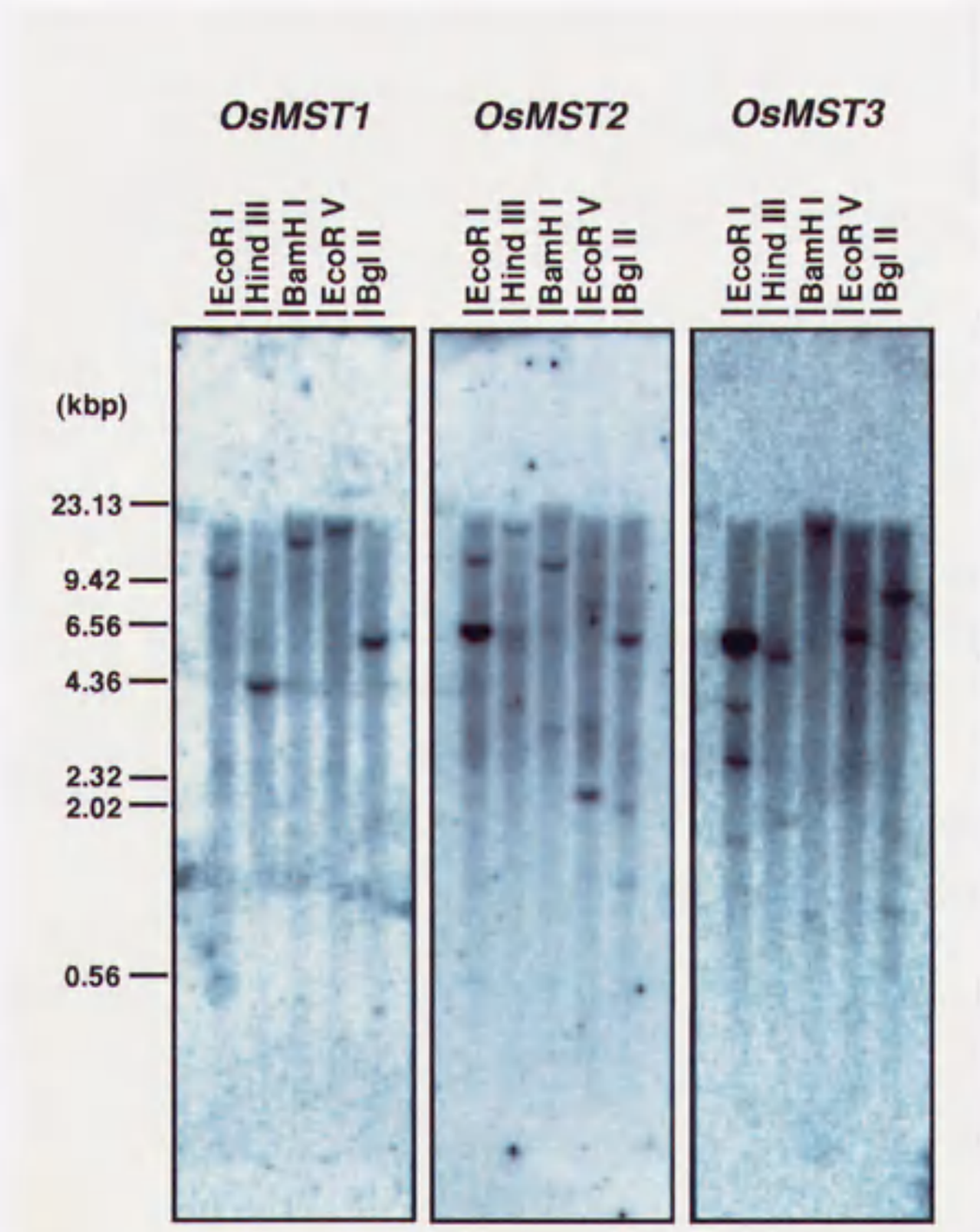

Figure 4-3 Southern blot analysis of *OsMST1-3* genes in rice genomic DNA.

Genomic DNA (2 μg) of each lane was digested with restriction enzymes and electrophoresed on 0.7 % agarose gel and blotted onto nylon membranes under alkaline conditions. Membranes were hybridized with the radiolabeled cDNA probes. After the wash, membranes were exposed using a Fujix BAS2000 Bio-Imaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

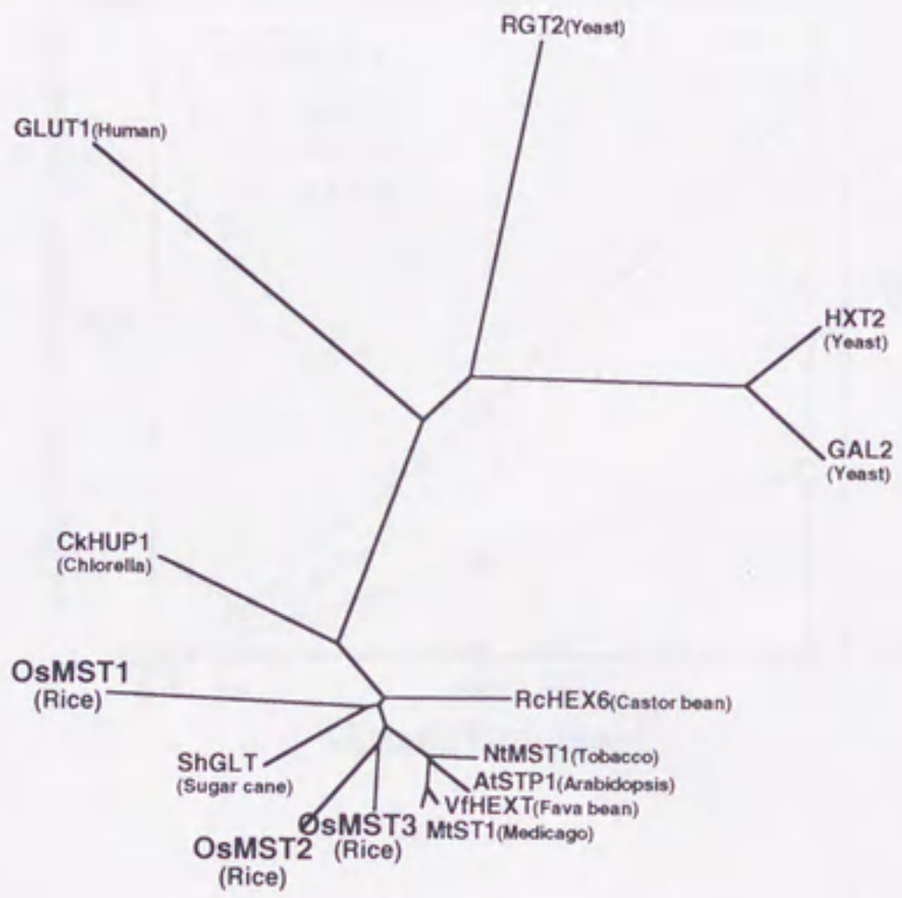

Figure 4-4 Phylogenetic tree of fourteen monosaccharide transporters.

The multiple-sequence alignment was constructed with the ClustalW and the TreeView program was used to generate the image.

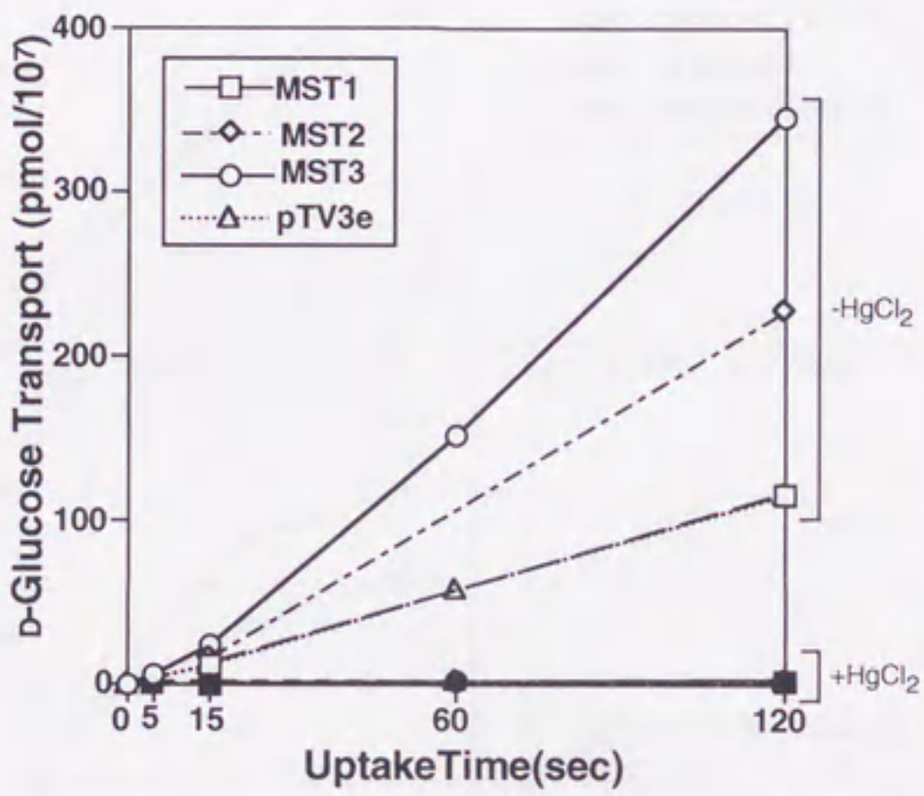

Figure 4-5 Glucose transport in yeast cells possessing the OsMST1-3.

OsMST1-3 of *S. cerevisiae* was maximally expressed using the GAL2 promoter in a multicopy plasmid, pTV3e, in an LBY416. D-glucose transport in yeast cells possessing the OsMST1 (□), OsMST2 (◇), or OsMST3 (○), and vector only (△) are shown. D-glucose transport in the presence of 0.5 mM $HgCl_2$ in OsMST1 (■), OsMST2 (◆), or OsMST3 (●), and vector only (▲) indicates the background of transport. The final substrate concentration was 0.1 mM in all transport experiments.

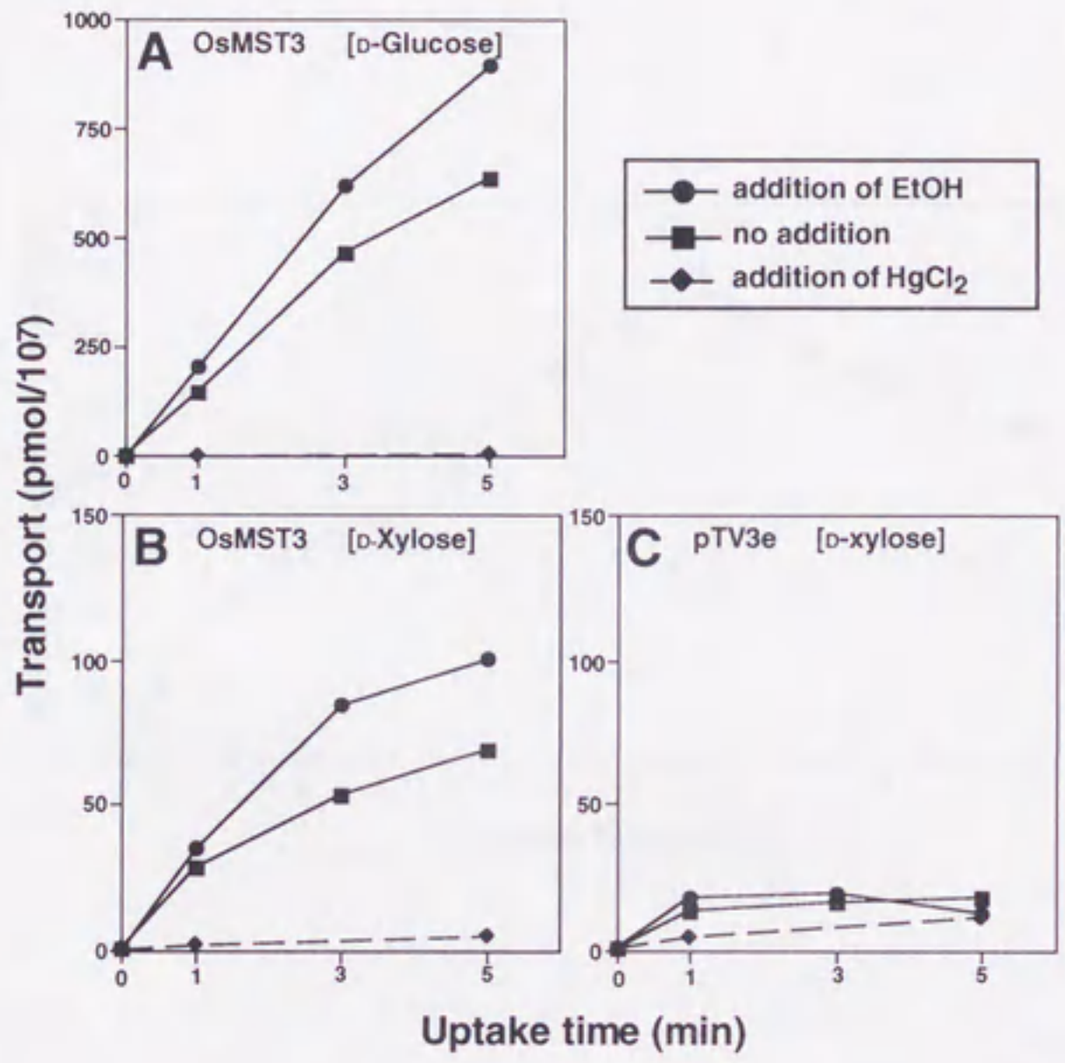

Figure 4-6 Transport of D-glucose and D-xylose in yeast cells possessing the OsMST3 (**A**, **B**) and D-xylose transport in the cells possessing vector only (**C**).

Transport in yeast cells with or without additional energization by 100 mM ethanol show (●) and (■), respectively. The transport in yeast cells in the presence of 0.5 mM $HgCl_2$ is shown by (◆).

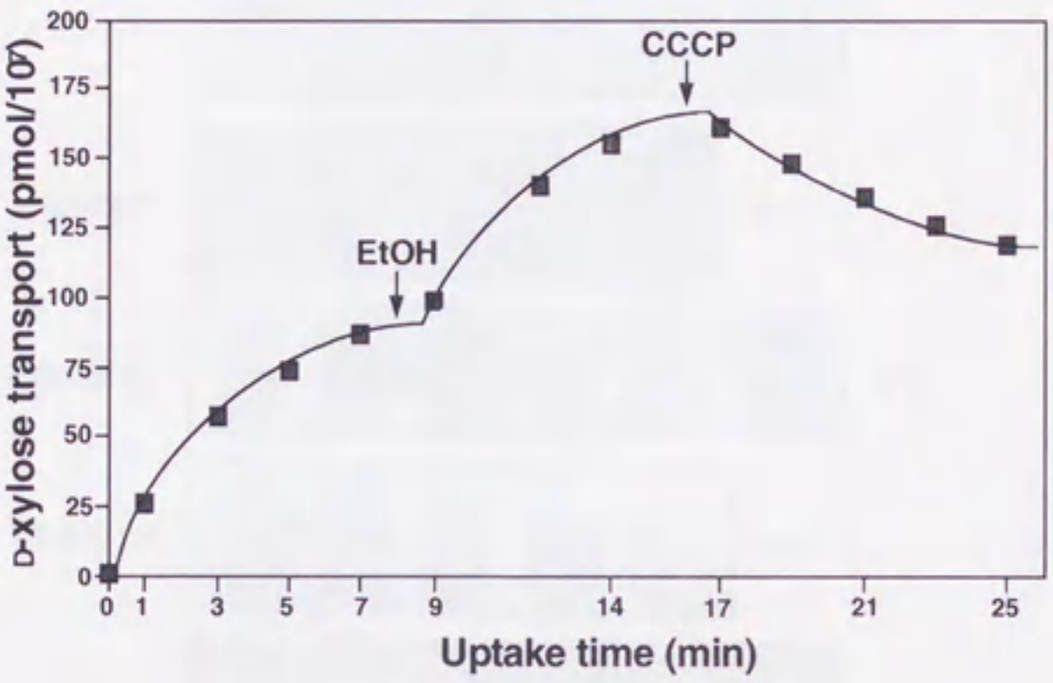

Figure 4-7 Effects of ethanol and uncoupler (CCCP) on D-xylose transport in yeast cells possessing the OsMST3.

The starting concentration of D-xylose in the medium was 0.1 mM, and the final concentration of CCCP in the medium was 50 μM. Arrows indicate the time of energization with ethanol (100 mM) and the addition of CCCP.

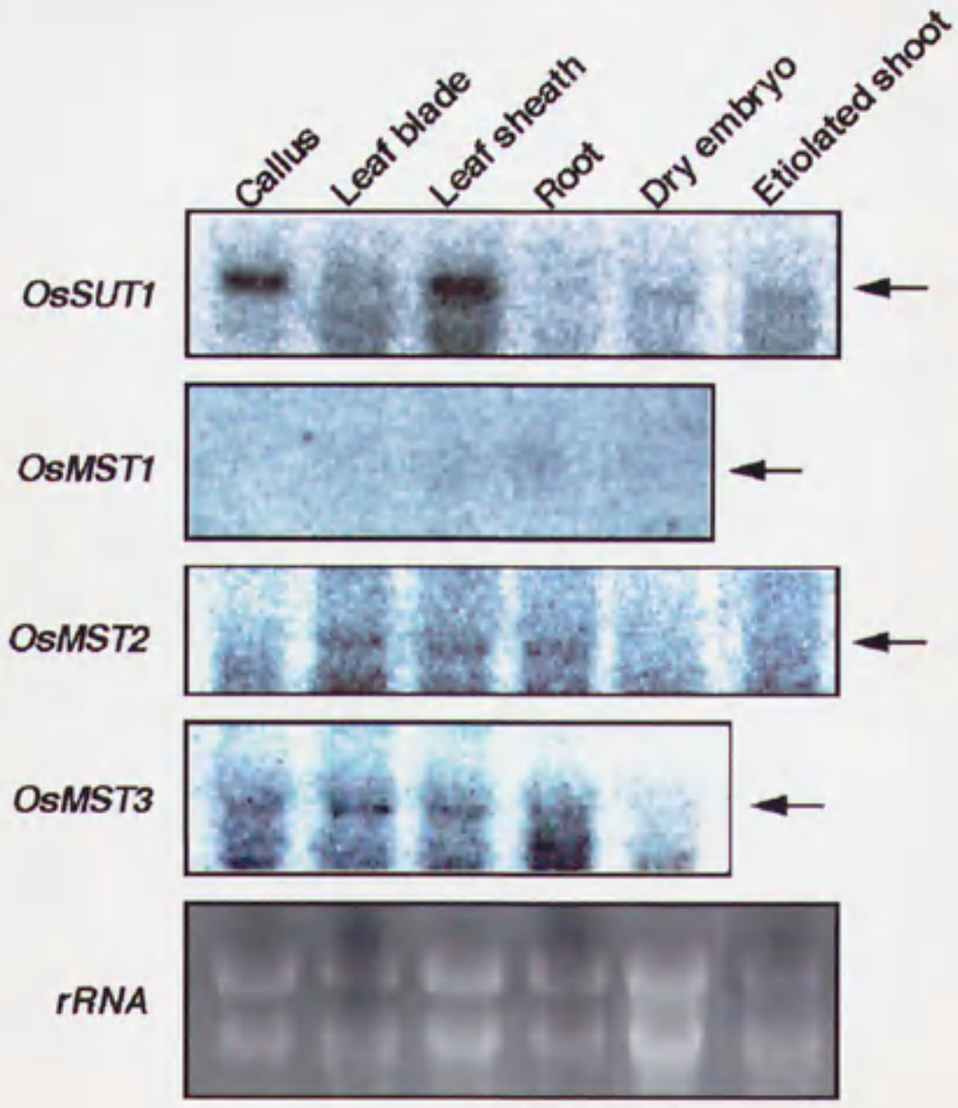

Figure 4-8 Northern blot analysis of *OsSUT1* and *OsMST1-3* mRNAs.

Total RNA (15 μg) of each sample was electrophoresed on formaldehyde gel and blotted onto nylon membranes and then hybridized with the radiolabeled cDNA probes. After the wash, membranes were exposed using a Fujix BAS2000 Bio-Imaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). Arrows represent mRNA signal of the probes. Staining with ethidium bromide is shown in the panel, rRNA.

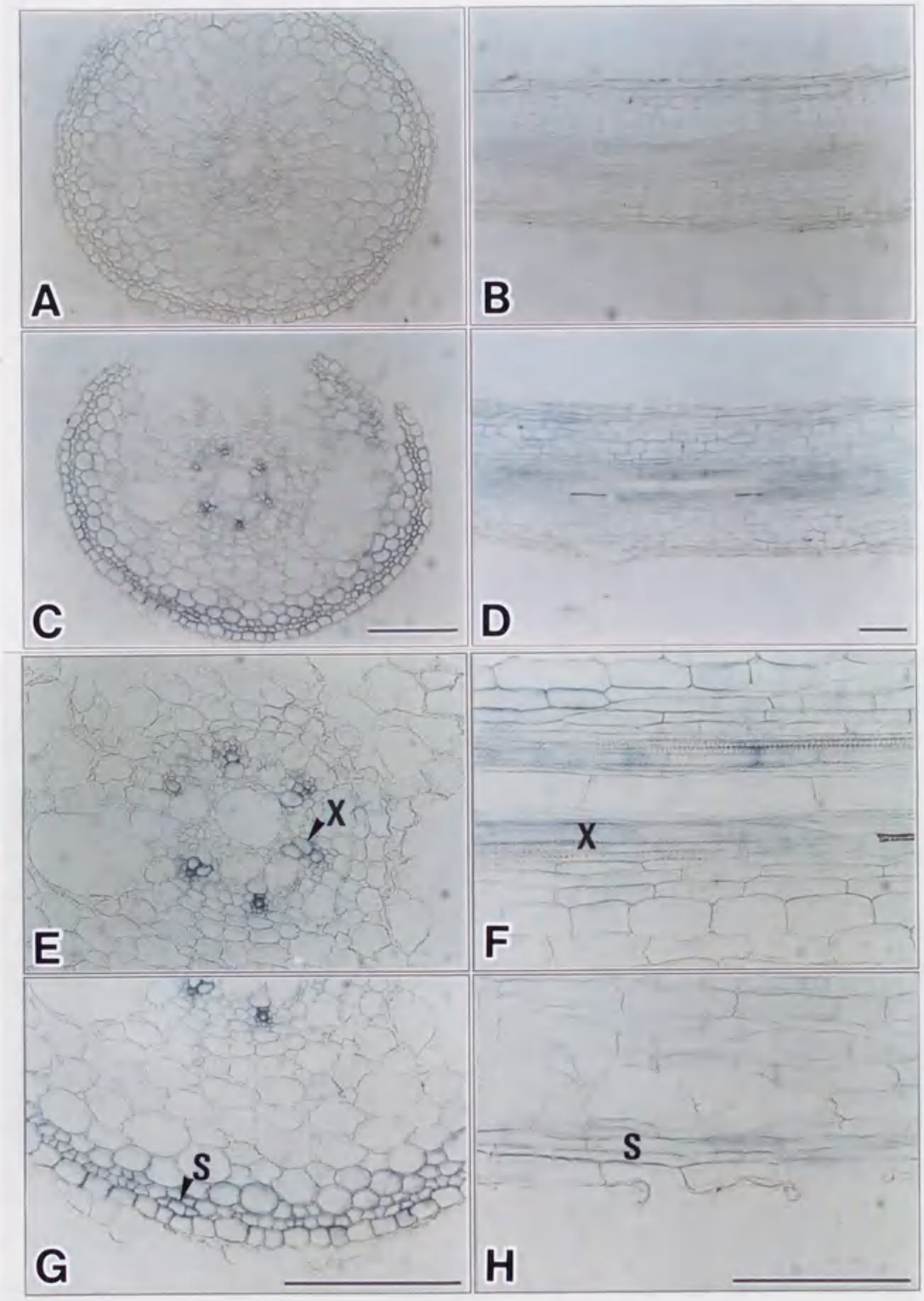

Figure 4-9 Localization of *OsMST3* mRNAs in rice root by *in situ* hybridization.
A and **B** *In situ* hybridization using digoxygenin-labeled sense probes. **C** to **H** *In situ* hybridization using digoxygenin-labeled antisense probes. Transverse sections and longitudinal sections are shown in **A** to **G** and **B** to **H**, respectively. Signals were detected in xylem cells (**E** and **F**) and in sclerenchyma cells (**G** and **H**).
X, xylem; S, Sclerenchyma. Bar: 0.1 mm.

Chapter 5

General Discussion

In this dissertation I analyzed the regulatory mechanisms of sugar transport and signaling, including the relation to hormonal effects. In the phloem of plant, the high osmotic pressure generated by energy-dependent sucrose accumulation and transpiration stream drive the long-distance flow of resources. Certainly, sucrose is the most important metabolite in this resource allocation system in most plants because it is generally the major end product of photosynthetic carbon metabolism. This system of sugar transport from the photosynthetic source to non-photosynthetic, heterotrophic sink tissues is known as assimilate partitioning. Consequently, sucrose represent a main phloem loader as energy and carbon resources for sink tissues, and sugars also, on the other hand, serve as signaling molecules whose transduction pathways influence developmental and metabolic processes [Koch 1996]. Indeed, a wide range of genes is regulated by sucrose and monosaccharides [Thomas and Rodriguez 1994, Koch 1996].

I analyzed rice α-amylase *RAmy3D* gene regulation by sugar in Chapter 2 and revealed the *cis*-acting elements involved in the sugar repression of the *RAmy3D* promoter activity. I finally demonstrated consensus nucleotide sequences designated as G and GATA motifs are important for the sugar repression of the gene. Morita et al. (1998) reported that the one of rice α-amylase isoform gene *RAmy1A*, which is clearly under GA control in the aleurone cells [Itoh et al. 1995], is also affected by the sugar regulation but to a smaller extent when compared with that of *RAmy3D*. Although G motif could not be found in the promoter sequence of the *RAmy1A* gene, whereas GATA motif, which is resided in TATCCT T/C motif (the so-called amylase element) as its antisense direction, can be found in the both α-amylase gene promoter sequences but one GATA sequence in *RAmy1A* opposed to two of *RAmy3D*. These result give me a expectation that this number of GATA sequence included might be caused to the difference of reduction of gene expression by sugar between *RAmy1A* and *RAmy3D*.

Many reports have indicated that the G motif sequence resides in the promoters of many genes that are switched on in response to diverse stimulatory

pathways, i.e. light, anearobiosis, *p*-coumaric acid and phytohormones such as ABA, ethylene and methyl jasmonate [Menkens et al. 1995]. I found that the *RAmy3D* promoter region has an ACGT sequence in G motif, responsible for its glucose repression, which is also found in the promoter region of ABA-regulated genes as part of the ABA-responsive elements (ABREs). Moreover, my data revealed that glucose depletion results in an increased ABA content in the rice embryos, speculating that ABA is involved in the glucose repression of the *RAmy3D*. As shown in Chapter 3, however, the promoter activity as well as mRNA transcription of *RAmy3D* gene did not show any affect by exogenous ABA, indicating that increased endogenous ABA content triggered by glucose depletion has no relation to the promotion of the *RAmy3D* promoter activity.

The rice *Rab16A* is one of the *Lea*-related gene identified as an typical ABA-inducible gene and the mRNAs and proteins accumulate in the seed during the late stage of seed development [Yamaguchi-Shinozaki et al. 1989, Ono et al. 1996], and has an ABRE with core sequence ACGT in motif I in its promoter region [Skriver et al. 1991]. I demonstrated that glucose repression of the *Rab16A* gene takes place both at the transcriptional and post-transcriptional level. This is consistant with the report of Perata et al. (1997) that the mRNA level of *Rab16A* was drastically reduced by additional glucose. Chen and Yu (1998) revealed the stability of mRNA is a critical factor related to sugar regulation of rice α-amylase gene *αAmy3* (identified as *RAmy3D* in my study) and the stretch of a 9-bp AU rich conserved sequence in 3' untranslated region might be related to the stability. I also demonstrated in Chapter 3 that the sugar repression of the *Rab16A* resulted in the destabilization of the mRNA by glucose. Further analysis will be needed to confirm the sequence for mRNA stability in response to sugar.

Increasing evidence indicates the existence of cross-talk between hormone and sugar signaling, but it is still unknown about the interaction between them. In rice, modulation of α-amylase genes by sugars and other metabolites is well described [Hwang et al. 1998, Umemura et al. 1998, Yu et al. 1996]. In this

dissertation I have revealed that ABA-inducible *Rab16A* gene is also modulated by sugar as I mentioned above.

From the results in Chapter 2 and 3, I could set up a hypothesis that the *trans*-acting factors have responsivility to both glucose and ABA signaling. Variable border sequence of ACGT in the promoter region of both sugar-repressive *RAmy3D* and *Rab16A* genes might be responsible for distinct response to ABA by either the putative *trans*-factor(s), possibly a basic region leucine zipper (bZIP) protein, bind or do not bind to the *cis*-element. Indeed, Rook et al. (1998) reported that a bZIP transcription factor in *Arabidopsis* was required to be involved in glucose signaling process(es).

In source tissue in higher plants, carbohydrates are produced by photosynthesis or starch degradation by amylolytic enzymes such as α-amylase. The carbohydrate is loaded into phloem cells to photosynthetically inactive sink organs by energy-dependent active transport system, i.e., sucrose transporter play a critical role on this apoplastic phloem loading. Unloaded sucrose in sink tissue is hydrolyzed by apoplastic invertase and then hexoses are taken up into cells by hexose (monosaccharide) transporter, besides the direct uptake of sucrose by sucrose transporter.

From the first glucose transporter gene is cloned from *Chlorella kessleri* [Sauer and Tanner 1989], a large number of monosaccharide transporters were isolated from lower and higher plants and were dissected the physiological and molecular complexity of plant sugar transporters. In Chapter 4, I described the first cloning of full-length cDNA clones of the monosaccharide transporter gene from rice. Plant monosaccharide transporters were isolated from many kind of dicotyledonous plants and well described for their characteristics and functions. In *Arabidopsis thaliana*, the genes of monosaccharide transporter make a large family, containing in excess of 26 genes [Lalonde et al. 1999]. For example, *AtSTP* (*Arabidopsis thaliana* sugar transport protein) genes, *1*, *2* and *4*, are highly expressed in sink organs; green pods and young leaves for *AtSTP1*, pollen for

AtSTP2, root tips and anthers for *AtSTP4* [Sauer et al. 1990, Truernit et al. 1996, 1999]. Furthermore, in the case of *AtSTP4*, the expression is enhanced in response to environmental stresses to deal with the increased carbohydrate demand of cells [Truernit et al. 1996]. On the other hand, there are only a few genes are cloned from monocotyledonous plants, and there have been no genes isolated from cereal plants up to now but I reported rice monosaccharide transporter clones in this dissertation. From the results of heterologous expression using *S. cerevisiae*, I demonstrated that OsMST3 protein is the energy-dependent monosaccharide transporter, possibly as a H^+ symporter. Through further characterization by Northern blot and *in situ* hybridization analysis, I found the *OsMST3* might play an important role on accumulation of monosaccharides required for cell wall synthesis at the stage of cell thickness. Immediately after the germination, plant must develop its vascular bundle to acquire nutrition as energy for further prompt growth. Sclerenchyma and xylem cells serve as supporting tissues to protect the vascular bundle from mechanical stresses, therefore cellulose is actively synthesized in the cell wall during the immature stage. From this point of view, these cells regard as 'sink' at the stage of cell thickness.

Özcan et al. (1996) reported that two unusual glucose transporters in *S. cerevisiae* appear to function as low- and high- glucose sensors that generate an intracellular glucose signal, one for induction, and one for repression of gene expression. Low levels of glucose bind to the Snf3 (high-affinity glucose sensor (receptor)) and high levels of glucose bind to the Rgt2 (low-affinity glucose sensor (receptor)), allowing maximum induction of several hexose transporter genes (*HXTs*) and this efficient system is needed for yeast and unicellular organisms to live and rapidly adapt to a variable environment. A putative sugar sensor in plant cells may be hexokinase [Jang and Sheen 1997, Umemura et al. 1998]. But it would be worthwhile to hunt for sugar sensor similar to Snf3 and Rgt2 in multicellular organisms like higher plants, which consist of mosaics of autotrophic green and heterotrophic non-green cells. How cells sense sugars is essential but

unsolved question. It is conceivable however, that cell has a spectrum of carriers of verifying affinity and capacity for sugar. Our understanding of plant sugar transporters has advanced considerably over the last 10 years. Further advances should provide dissections of regulatory pathways that control transporters and better understanding of their important contributions to plant growth and development.

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genes and metabolic activities in germinating cereal grains. *Plant Mol. Biol.* **30**: 1277-1289.

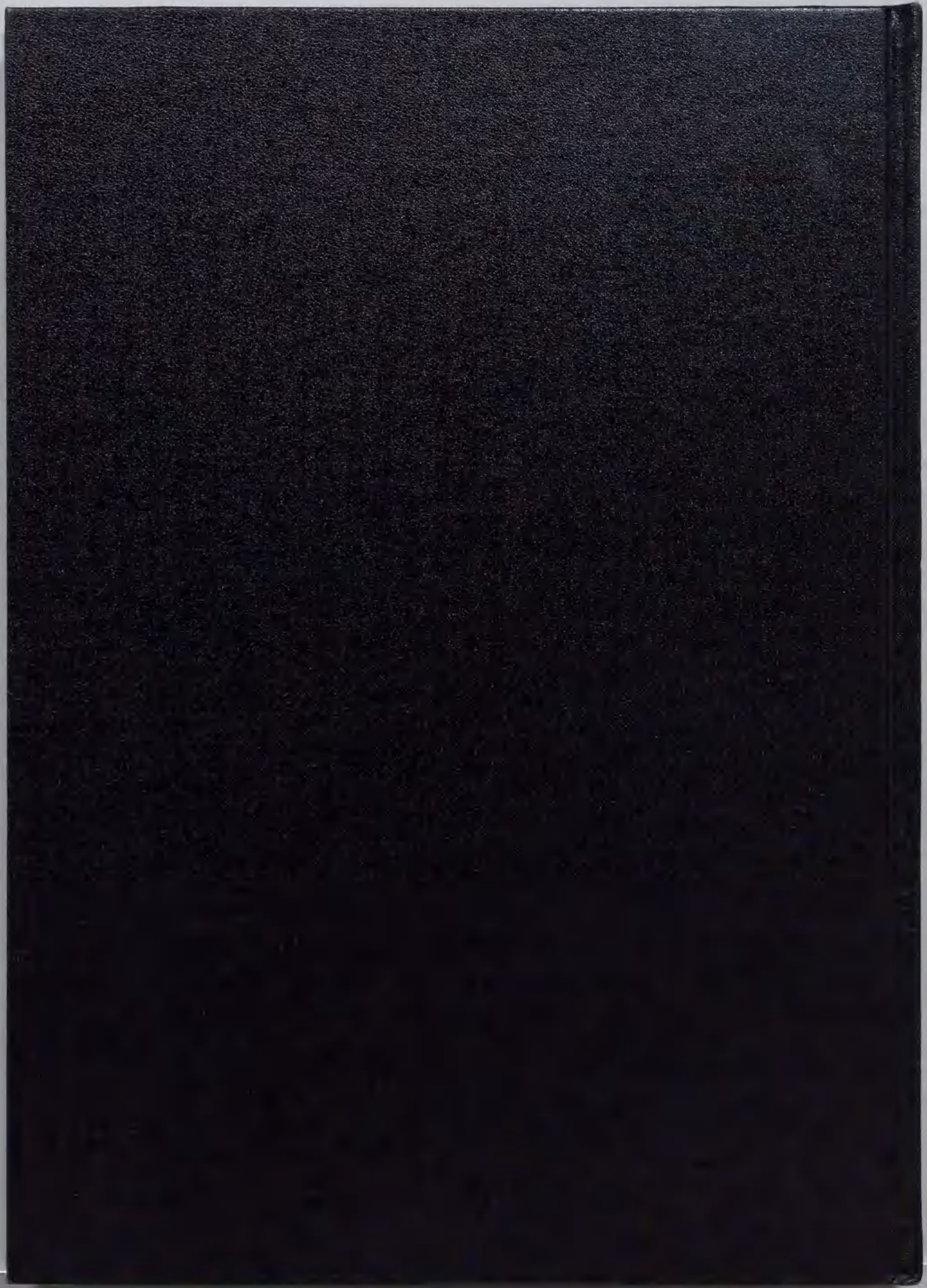
List of Publications

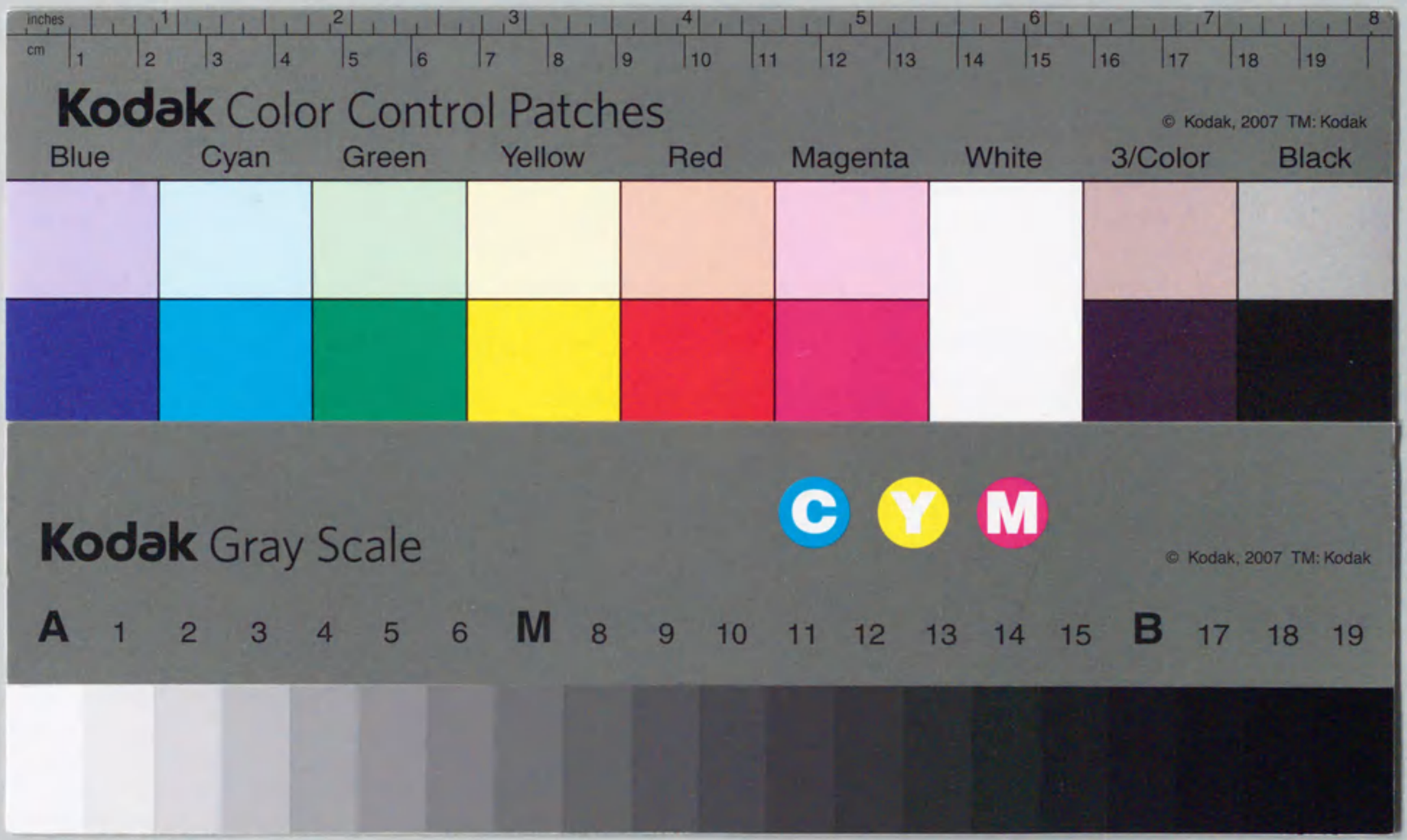
(1) **Toyofuku K, Umemura T and Yamaguchi J** (1998) Promoter elements required for sugar-repression of the *RAmy3D* gene for α-amylase in rice. *FEBS Lett.* **428**: 275-280.

(2) **Toyofuku K and Yamaguchi J** (1998) Abscisic acid dose not affect sugar-repression of rice α-amylase gene, *RAmy3D*. *Rice Genet. Newslett.* **15**: 173-175.

(3) **Toyofuku K, Loreti E, Vernieri P, Alpi A, Perata P, and Yamaguchi J** (2000) Glucose modulates the abscisic acid-inducible *Rab16A* gene in cereal embryos. *Plant Mol. Biol.* in press

(4) **Toyofuku K, Kasahara M and Yamaguchi J** (2000) Characterization and expression of monosaccharide transporters (*OsMSTs*) in rice. *Plant Cell Physiol.* submitted

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